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DNA Damage, Homology-Directed Repair, and DNA Methylation

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To explore the link between DNA damage and gene silencing, we induced a DNA double-strand break in the genome of Hela or mouse embryonic stem (ES) cells using I-SceI restriction endonuclease. The I-SceI site lies within one copy of two inactivated tandem repeated green fluorescent protein (GFP) genes (DR-GFP). A total of 2%–4% of the cells generated a functional GFP by homology-directed repair (HR) and gene conversion. However, ~50% of these recombinants expressed GFP poorly. Silencing was rapid and associated with HR and DNA methylation of the recombinant gene, since it was prevented in Hela cells by 5-aza-2'-deoxycytidine. ES cells deficient in DNA methyl transferase 1 yielded as many recombinants as wild-type cells, but most of these recombinants expressed GFP robustly. Half of the HR DNA molecules were de novo methylated, principally downstream to the double-strand break, and half were undermethylated relative to the uncut DNA. Methylation of the repaired gene was independent of the methylation status of the converting template. The methylation pattern of recombinant molecules derived from pools of cells carrying DR-GFP at different loci, or from an individual clone carrying DR-GFP at a single locus, was comparable. ClustalW analysis of the sequenced GFP molecules in Hela and ES cells distinguished recombinant and nonrecombinant DNA solely on the basis of their methylation profile and indicated that HR superimposed novel methylation profiles on top of the old patterns. Chromatin immunoprecipitation and RNA analysis revealed that DNA methyl transferase 1 was bound specifically to HR GFP DNA and that methylation of the repaired segment contributed to the silencing of GFP expression. Taken together, our data support a mechanistic link between HR and DNA methylation and suggest that DNA methylation in eukaryotes marks homologous recombined segments.

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Introduction

Cellular metabolites and exogenous DNA-damaging agents constantly modify DNA. Such modifications may induce death in unicellular organisms or degenerative changes and aging in multicellular organisms. DNA damage can also activate or amplify biochemical pathways that regulate cell growth and division or coordinate DNA replication with DNA repair. Of the various forms of DNA damage, the most hazardous to the cell are DNA double-strand breaks (DSBs). DSBs are generated when the two complementary strands of the DNA double helix are broken simultaneously at nearby sites. Physically dissociated ends can recombine inappropriately with other genomic sites, leading to chromosomal translocations.

Because of threats posed by DSBs, eukaryotic cells have evolved complex, highly conserved systems to rapidly and efficiently detect these lesions, signal their presence, and bring about their repair. DSBs are principally repaired either by homologous recombination (HR) or by nonhomologous end joining (NHEJ). These pathways are largely distinct from one another and function in complementary ways [1,2]. In HR, the damaged chromosome synapses with and retrieves genetic information from an undamaged DNA partner with which it shares extensive sequence homology. In contrast,

NHEJ involves ligation of two DNA ends and does not entail a synapse of broken DNA with an undamaged DNA molecule.

HR can lead to gene conversion, a conservative process in which a donor DNA sequence with homology to both sides of the DSB supplies genetic information to repair the DSB [3,4]. The homologous sequence is copied into the broken locus,

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Abbreviations: 5-Aza-2'-deoxycytidine; CMV, cytomegalovirus; Dnmt1, DNA methyl transferase 1; DR, tandem repeated; DS, double-strand break; ES, embryonic stem; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; HR, homology-directed repair or homologous recombination; HR-H, high expressing; HR-L, low expressing; LOH, loss of heterozygosity; NHEJ, nonhomologous end joining; qPCR, quantitative PCR performed on DNA; RT-PCR, reverse-transcribed PCR performed on RNA samples

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Author Summary

Genomic DNA can be modified by cytosine methylation. This epigenetic modification is layered on the primary genetic information and can silence the affected gene. Epigenetic modification has been implicated in cancer and aging. To date, the primary cause and the mechanism leading to DNA methylation are not known. By using a sophisticated genetic system, we have induced a single break in the double helix of the genomes of mouse or human cells. This rupture was repaired by a very precise mechanism: the damaged chromosome pairs and retrieves genetic information from an undamaged and homologous DNA partner. This homology-directed repair was marked in half of the repaired molecules by *de novo* methylation of cytosines flanking the cut. As a direct consequence, the gene in these repaired molecules was silenced. In the remaining molecules, the recombinant DNA was undermethylated and expressed the reconstituted gene. Since homology-directed repair may duplicate or delete genetic information, epigenetic modification of repaired DNA represents a powerful evolutionary force. If the expression of the repaired gene is harmful, only cells inheriting the silenced copy will survive. Conversely, if the function of the repaired gene is beneficial, cells inheriting the under-methylated copy will have a selective advantage.

making the repaired locus an exact copy of donor sequence, without altering the donor sequence.

DNA methylation is a covalent postreplicative modification of genomic DNA. It has been implicated in development and differentiation [5], X chromosome inactivation [6], imprinting [7], and cancer [8,9]. In mammals methylation, promoted by DNA methyltransferases, occurs mostly on the cytosine residues of CpG dinucleotides. DNA methylation induces and maintains a transcriptionally silent state of genes [10]. DNA methylation can directly interfere with transcription by preventing binding of basal transcriptional machinery or ubiquitous transcription factors that require contact with cytosine in the major groove of the double helix. A total of two types of DNA methylation can be distinguished. Stable methylation is inherited through generations in a male- or female-specific fashion and is responsible for both mono- and biallelic imprinting. Metastable methylation is variable and generates different methylation patterns among individual cells and cell types. It is modified by environment and changes during the lifetime of individual cells. Metastable methylation as the consequence of DNA damage repair is the subject of this study.

Several reports support an association between DNA damage, recombination, and DNA methylation: (1) DNA methyltransferase and proliferating cell nuclear antigen, an auxiliary factor in DNA replication and repair, form a complex *in vitro* [11]; (2) DNA methyltransferase 1 (Dnmt1) is recruited to DNA repair sites [12]; (3) Mouse cells lacking Dnmt1 are genetically unstable [13,14]; (4) Global inhibition of methylation leads to genome instability in human cell lines [15].

We explore this association using the mammalian system pioneered by M. Jasin [16,17] in which recombination between partial duplications is initiated by a specific DSB in one copy. Recombination products can be detected by direct analysis of the DNA flanking the DSB or by the appearance of the product of the recombined gene. We show that gene conversion profoundly modifies the methylation

pattern of the repaired DNA and that this methylation silences the recombined gene. We also report that Dnmt1 was specifically associated to the chromatin of homologous repaired green fluorescent protein (GFP).

We propose that the DNA methylation, induced by HR, marks the repaired DNA segments and protects cells against unregulated gene expression following DNA damage.

Results

To explore the molecular mechanism(s) linking DNA damage and gene silencing, we induced a DSB at a specific DNA sequence in the genome and monitored recombination and expression of the recombinant gene. We also examined the structure and methylation pattern at the DSB locus following repair. Finally, we asked if inhibiting DNA methylation affected recombination and/or expression of the recombinant product.

Recombination Assay

Our recombination assay relies on the two inactivated tandem repeated (DR)-GFP plasmid developed by M. Jasin [16], which contains two mutated GFP genes oriented as direct repeats and separated by a drug selection marker, the puromycin N-acetyltransferase gene (Figure 1A). An upstream cytomegalovirus (CMV) enhancer fused to the chicken β -actin promoter provided a strong and insulated transcriptional unit (see Materials and Methods for the structure of DR-GFP). The upstream (5') GFP gene (cassette I) carries a recognition site for I-SceI, a rare-cutting endonuclease that does not cleave several eukaryotic genomes tested [17]. The I-SceI recognition sequence was incorporated into a naturally occurring BclI restriction site by substituting 11 bp of the wild-type gene. These substituted base pairs supply two in-frame stop codons that terminate translation, thereby inactivating cassette I. The downstream (3') GFP (cassette II) is inactivated by upstream and downstream truncations, leaving only ~502 bp of GFP (see Materials and Methods for the structure of DR-GFP). Hela cells were stably transfected with DR-GFP and selected in the presence of puromycin. Puromycin-resistant pools of cells carrying DR-GFP at various loci were then transiently transfected with a vector expressing I-SceI. The resultant DSB induced homologous recombination. Fluorescence-activated cell sorter (FACS) analysis was used to reveal the percentage of cells expressing GFP (Figure 1A, upper panel). We maintained the cells after transfection in the presence of puromycin to eliminate recombinant clones generated by homology-mediated deletion [16].

The structure of the GFP locus was determined by PCR analysis and Southern blot (see below). We used a 3' end primer present only in cassette I but not in cassette II. The two 5' primers, unrec, amplified nonrecombinant units, and rec amplified only recombined units (Figure 1A). The rec primer amplified a 436-bp fragment 48 hours after I-SceI transfection, (Figure S1A). This PCR product indicates a wild-type GFP gene generated by gene conversion at the I-SceI site. In contrast to the unrec PCR product (438 bp), the rec product was detected only after exposure of cells to I-SceI.

We then measured the expression of GFP mRNA by reverse-transcribed (RT)-PCR (Materials and Methods). Recombined GFP mRNA was detected only in cells transfected

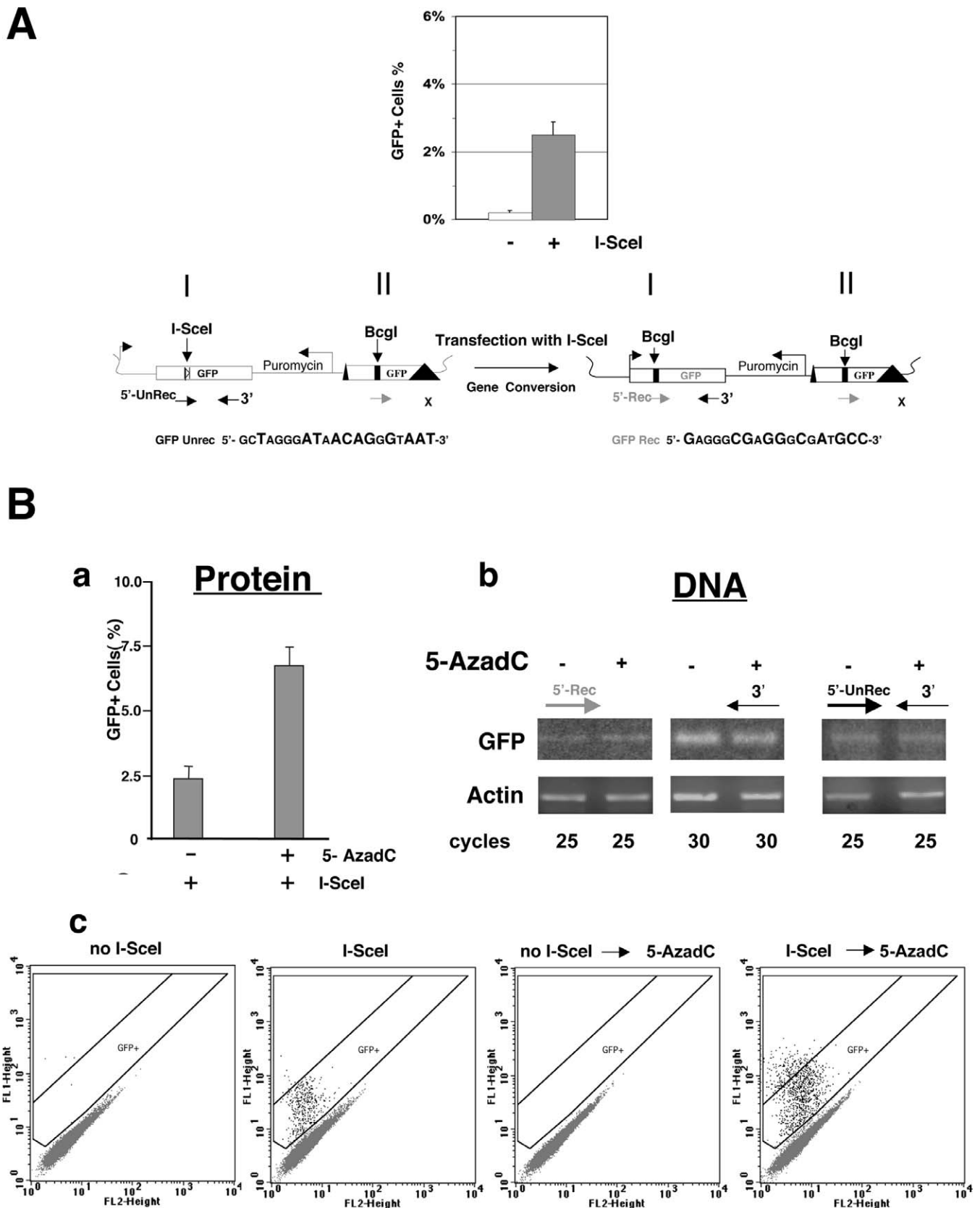


Figure 1. DNA Methylation and Recombination

(A) In the recombination assay primers for PCR and RT-PCR amplification were: (1) 5'-unrec (unrecombinant) centered on the I-SceI site, present only in cassette I and (2) 5'-rec (recombinant) centered in the BclI site present only in the converted GFP or in cassette II. The 3' end primer (3') is located in

cassette I in a sequence deleted in cassette II (X). The 5'-rec primer can pair with the 3' primer only after its reconstitution in the 5' cassette by gene conversion. The sequence of the 5' primers is indicated with the distinguishing bases in capital letters.

Stable HeLa cells containing the DR-GFP plasmid were transiently transfected with I-SceI and pSVβGal (Promega) expression vectors as described in Materials and Methods. After 3 d, GFP cells were scored by FACS. The histogram shows data from three experiments. Transfection efficiency in these three experiments was $70\% \pm 5\%$.

(B) Inhibition of methylation reveals silenced recombinants. HeLa cells carrying the DR-GFP plasmid were transfected with I-SceI and pSVβGal expression vectors and treated with $5 \mu\text{M}$ 5-AzadC 48 h posttransfection, as described in Materials and Methods. (a) GFP⁺ cells were analyzed by FACS. The ordinate shows the fraction of GFP⁺ cells over total cells transfected. The efficiency of transfection was $70\% \pm 5\%$. To determine the effect of 5-AzadC on GFP fluorescence, we compared the percentage of GFP⁺ cells before and after 5-AzadC treatment in six experiments (three in duplicate) with a nonparametric matched pairs test, such as the Wilcoxon sign-rank ($p < 0.016$). (b) For the DNA analysis GFP clones were analyzed by PCR with the primers indicated in Figure 1A. The results shown were derived from DNA of I-SceI treated cells (see Materials and Methods). (c) The cells were treated with 5-AzadC 48 h after transfection as described in Materials and Methods and analyzed by FACS. Treatment with 5-AzadC after I-SceI transfection significantly increased the number and the intensity of fluorescence of GFP⁺ cells, while 5-AzadC alone was ineffective. The area indicated as "GFP⁺⁺" includes 95% of GFP-expressing cells after I-SceI transfection.

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with I-SceI (Figure S1B). To verify the presence of bona fide recombined GFP mRNA, we cleaved the double-stranded cDNA prior to PCR with BcgI (Figure S1B, quality check). BcgI specifically cut the recombined GFP gene, ablating amplification with the rec primer.

Methylation Reduces GFP Expression but Not Recombination Frequency

DSBs are efficient substrates of homologous recombination. The low yield of GFP⁺ cells after DSB generation raised the possibility that some wild-type GFP recombinants were silenced, possibly by methylation. Accordingly, we asked if inhibiting methylation increased the yield of cells that expressed GFP. The HeLa cell DR-GFP pool was split two days after exposure to I-SceI. One group was treated with 5-aza-2'-deoxycytidine (5-AzadC) (see Materials and Methods) for 48 hours to block or reverse DNA methylation [18]. FACS analysis revealed that 5-AzadC significantly increased the number of GFP⁺ cells (Figure 1B).

5-AzadC did not enhance the yield of GFP⁺ cells by stimulating homologous recombination. PCR analysis, performed as described in Figure S1A, showed clearly that treatment with 5-AzadC after I-SceI exposure did not increase the number of GFP recombinant genes (Figure 1B [b]). This experiment was repeated with DNA and RNA derived from independent transfections with identical results and on isolated clones (Figure S2). The effects of 5-AzadC on the intensity of GFP fluorescence can be appreciated in the dot-plot shown in Figure 1B (c).

We considered the possibility that inhibition of recombinant GFP expression was not induced by homology-directed repair, but resulted instead from subsequent transgene silencing, often observed in cultured cells [19]. Accordingly, we monitored the expression of a wild-type GFP transgene driven by a CMV promoter (wild-type GFP) in cells transfected with the I-SceI vector and treated with 5-AzadC, as described in Figure 1B. Figure 2A shows that in contrast to the expression of recombinant GFP, which is bimodal in distribution, wild-type GFP expression is unimodal. Furthermore, unlike recombinant GFP, wild-type GFP expression was not enhanced by 5-AzadC.

To monitor the timing of silencing of recombinant GFP genes and to visualize the effect(s) of 5-AzadC, we separated high- (HR-H) and low-expressing (HR-L) cells, as shown in Figure 2B. The separated cells were grown for the indicated times, and parallel cultures were treated with 5-AzadC for 24 h. GFP expression was monitored by FACS. Figure 2C shows that: (1) only the HR-L fraction was silenced with time; (2)

silencing was rapid and reached a plateau two weeks after I-SceI transfection; (3) 5-AzadC stimulated GFP expression in the HR-L population at all time points tested but did not affect expression of the HR-H population. Wild-type GFP expression declined only slightly during the two-week period tested.

Analysis of Individual DR-GFP Clones

The data shown above suggest that recombination products induced by I-SceI cleavage are silenced by methylation. These results were obtained from pools of cells carrying DR-GFP integrated randomly in the genome and did not distinguish among individual clones. We therefore asked if the integration locus influenced the expression of GFP recombination products, and by inference, their methylation status. We isolated several HeLa DR-GFP clones with single insertions (Figure S3) and transfected them with I-SceI. Figure S3A (inserts) shows the fluorescent mean intensity as dot plots in red and the fraction of GFP-expressing cells in three individual clones. Both the frequency (ordinate) and the fluorescence intensity (abscissa) segregated in discrete peaks. We repeatedly transfected the individual clones with I-SceI and determined GFP fluorescence intensity after normalization for transfection efficiency. The results, shown in Figure 3B, indicate differences in GFP expression from experiment to experiment. In all cases, I-SceI induced GFP expression, and each clone displayed a particular range of GFP levels, probably due to the specific integration site.

We showed above that inhibition of methylation with 5-AzadC significantly increased the number of GFP⁺ cells in a pool of cells carrying DR-GFP at different loci (Figures 1B and 2C). We now asked if 5-AzadC affected GFP expression in an individual clone. FACS analysis of clone 3 shows that GFP⁺ recombinants appear only after I-SceI exposure (Figure 4A and 4B). Transient treatment with 5-AzadC prior to DSB formation did not increase the number of GFP positive cells (Figure 4B and 4C). As was the case with the pooled DR-GFP transfectants, 5-AzadC added after I-SceI transfection significantly enhanced the yield of cells expressing GFP at high levels (Figure 4D). The same experiments were performed with clones 1 and 2 with similar results (unpublished data). Note that in clones with a single integration site, 5-AzadC stimulates expression levels to the level of the HR-H average. This effect is not evident in the pool of DR-GFP clones (Figures 2C and 4D).

These results agree with those obtained from the pool of clones and indicate that methylation following homologous repair of DSBs suppresses expression of a fraction of

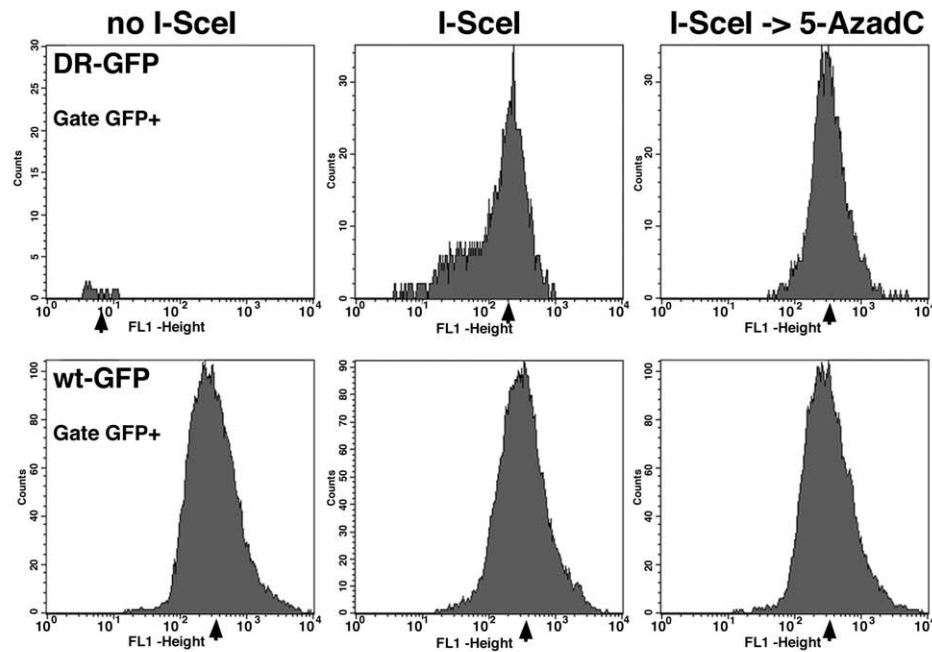
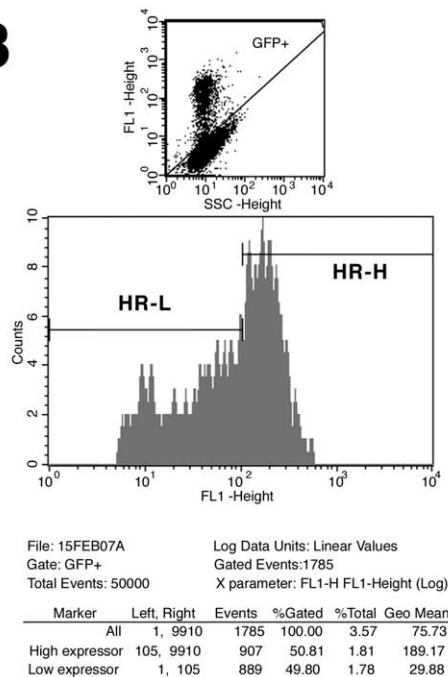
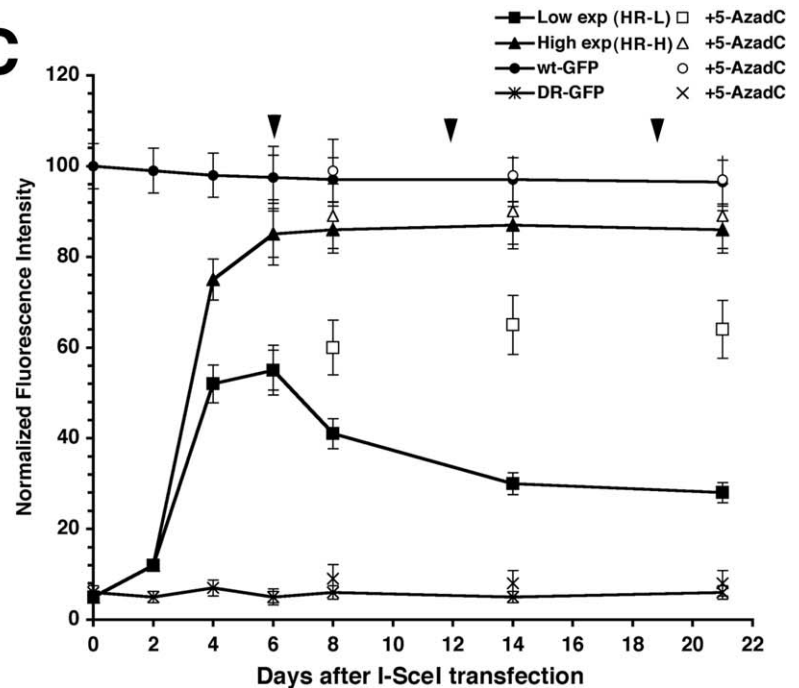
A**B****C**

Figure 2. GFP Silencing in Recombinant Clones

Hela cells carrying the DR-GFP or wild-type GFP plasmids were transfected with I-SceI and pSVβGal expression vectors, as described in Materials and Methods.

(A) Shown is the fluorescence analysis of cell lines stably expressing wild-type GFP. Treatment with 5-AzadC was carried out as described in Figure 1. The cells were analyzed 7 d following 5-AzadC treatment. The GFP⁺ gate includes 95% of GFP-expressing cells. The mean fluorescence (arrow) and the % of GFP⁺ cells were: (1) DR-GFP (no-I-SceI) 7.4 and 0.4%; (I-SceI) 203.56 and 4.7%; and (I-SceI+ 5-AzadC) 335.31 and 7.6%, respectively and (2) wild-type GFP (no-I-SceI) 357.51 and 95.4%; (I-SceI) 361.12 and 96.2%; and (I-SceI+ 5-AzadC) 389.68 and 89.6%, respectively.

(B) Hela cells carrying the DR-GFP plasmid were transfected with I-SceI and pSVβGal as indicated in Figure 1. GFP⁺ cells were sorted by FACS and divided in two pools 4 d after transfection: HR-L and HR-H GFP expressors. The upper panel shows the gate used to select GFP⁺ cells. The lower panel shows only GFP⁺ cells and the gates used for sorting HR-L and HR-H.

(C) GFP fluorescence in the mass culture was monitored by FACS before transfection and 2 d following transfection. Sorted cells were monitored at 4, 6, 8, 14, and 21 d after transfection. Parallel cultures at 6, 12, and 19 d posttransfection were treated for 24 h with 5-AzadC (arrows). 5-AzadC was washed away and GFP fluorescence was determined 24 h later. DR-GFP and wild-type GFP represent cell lines derived from DR-GFP pools transfected with pSVβGal plasmid or from a stable line expressing the wild-type GFP gene, respectively.

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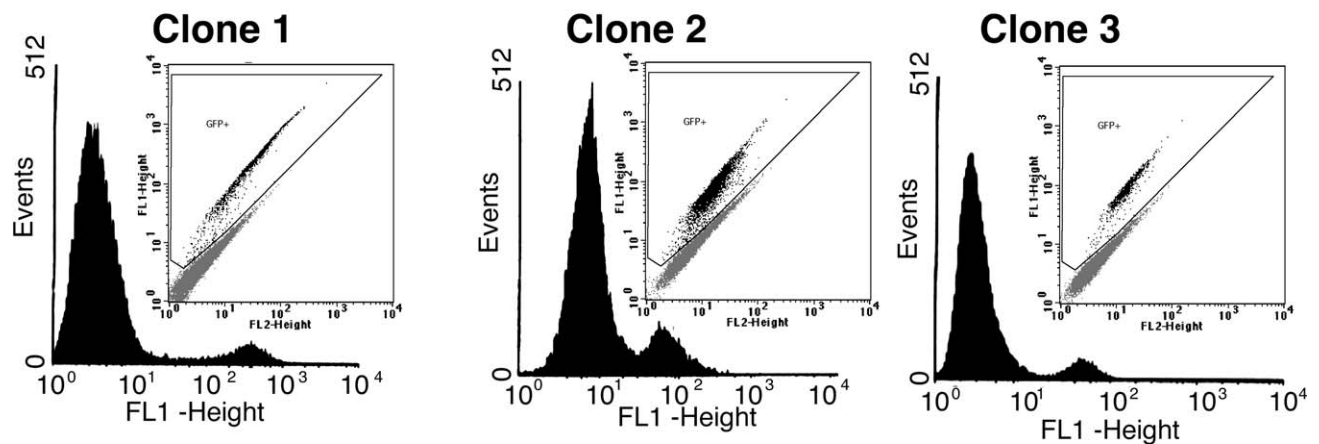
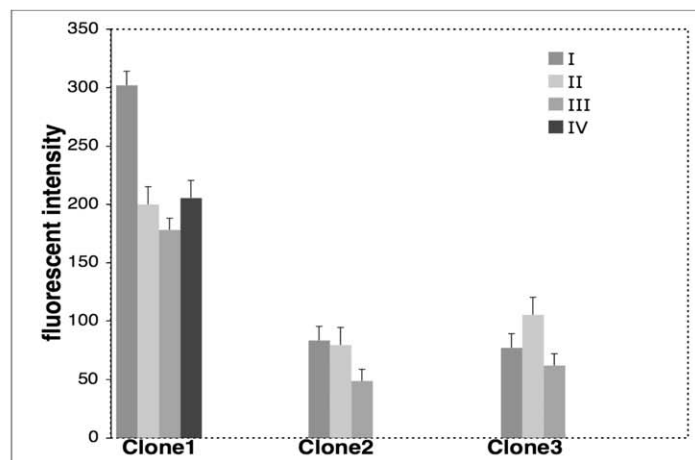
A**B**

Figure 3. Recombination in Individual DR-GFP Clones

Single clones were isolated from pooled cultures of Hela carrying inactive copies of DR-GFP. The three clones analyzed indicated as 1, 2, and 3 contained a single insertion with one or four copies of DR-GFP by Southern blot and qPCR (Figure S2). These clones were transfected in several independent experiments with I-SceI and pSVβGal vectors and scored for GFP⁺ cells.

(A) Shown is FACS analysis of three clones 72 h after I-SceI transfection. The ordinate shows the number of cells and the abscissa the intensity of the fluorescence, respectively. The inset shows the dot plot of the bivariate analysis.

(B) The experiment illustrated in (A) was repeated several times, and the mean of intensity of fluorescence of GFP⁺ cells is shown in the histogram. The roman numerals indicate an individual experiment.

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recombinant GFP genes. Additionally, the bimodal GFP expression distribution characteristic of the mass culture was also seen in clones carrying DR-GFP inserted at a single chromosomal location.

DNA Methyl-Transferase I Inhibits Expression of Recombinant GFP Genes

Stimulation of recombinant GFP gene expression by 5-AzadC suggested that a significant fraction of recombinant genes was silenced by methylation. We confirmed this conclusion in another system in which global methylation was impaired by inactivation of Dnmt1. Dnmt1 is responsible for methylation maintenance in the mouse genome [5]. We transfected a Dnmt1^{−/−} ES cell line [5] with DR-GFP. The pool of puromycin-resistant clones was then transfected with I-SceI and analyzed as described above for Hela cells. Our results indicate that the frequency of HR was the same in wild-type and Dnmt1^{−/−} ES cells, as shown by PCR and

quantitative (q)PCR (Figure 5A). FACS analysis indicates that the percentage of Dnmt1^{−/−} cells that expressed GFP at elevated levels was higher than wild-type cells (Figure 5B and 5C). Finally, treatment with 5-AzadC increased the fraction of wild-type ES high expressors but did not amplify the expression of GFP in Dnmt1^{−/−} cells (Figure 5B and 5C). These data suggest that Dnmt1-dependent methylation silences GFP expression in recombinant clones.

CpG Methylation of Individual Molecules before and after Repair

To visualize the methylation status of individual DNA molecules before and after DSB repair, we analyzed DNA of individual cells from the mass ES cell culture and determined the methylation profile at the I-SceI site. Genomic DNA was treated with bisulfite, which converts cytosine but not 5-methylcytosine to thymine, and then amplified by PCR. Cytosines detected by direct sequence analysis, therefore,

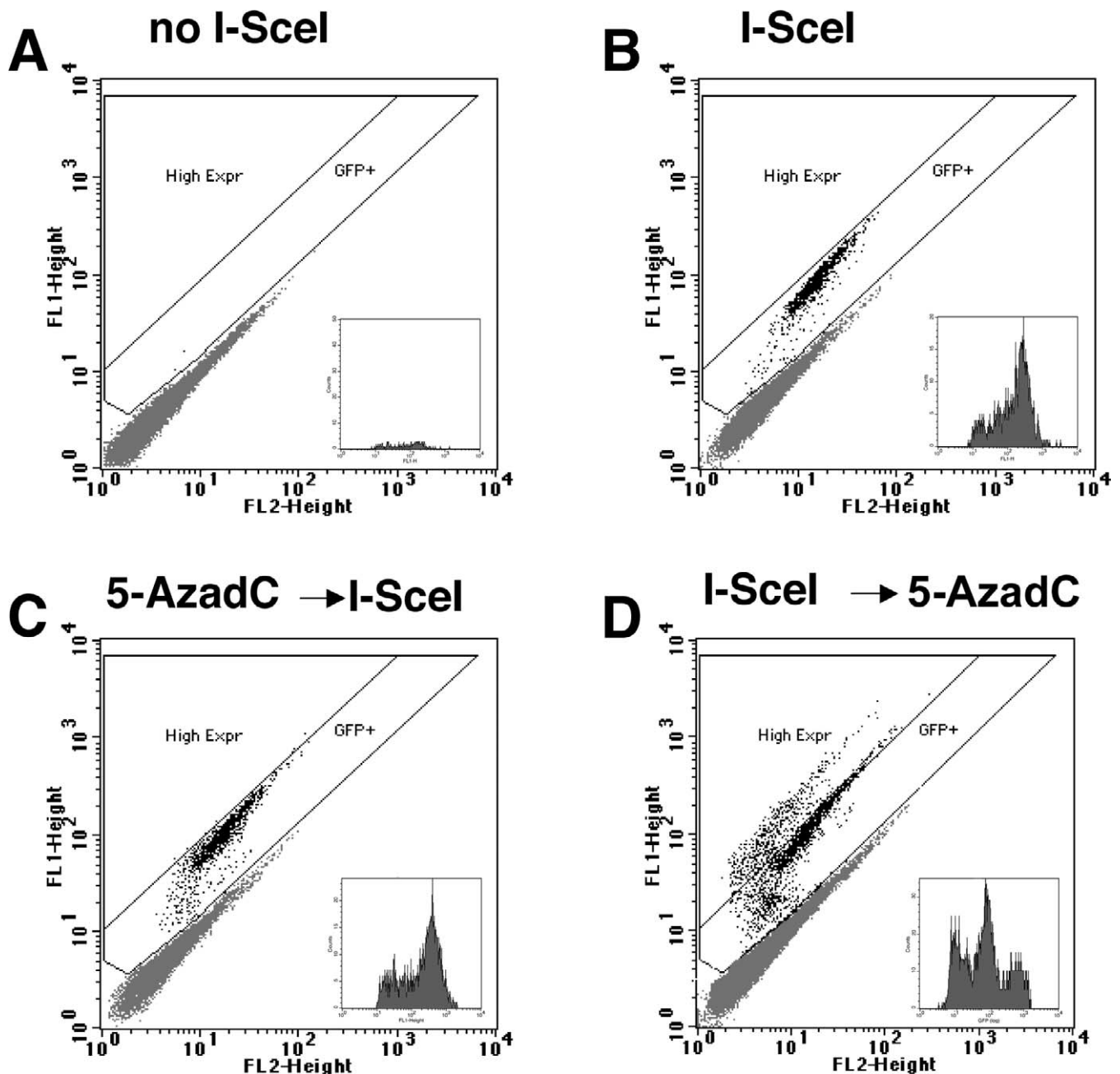


Figure 4. DNA Methylation Is Induced by I-SceI Cleavage/Recombination

Dot plots of GFP expression in clone 3 after I-SceI transfection are presented. The insets show the mean of fluorescence intensity versus number of GFP⁺ cells.

(A) Cells transfected with a control plasmid (pSVβGal) are shown.

(B) Cells transfected with I-SceI are shown.

(C) Cells treated with 5-AzadC 48 h before I-SceI transfection are shown.

(D) Cells transfected with I-SceI and treated with 5-AzadC 48 h later are shown. All FACS analyses were performed 5 d after transfection. The effects of 5-AzadC were analyzed also at 2–4 and 6 d after transfection, and the results were similar. The efficiency of transfection was 70% ± 10%. Experiments with transfection efficiency lower than 70% were excluded from the analysis.

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represent methylated residues. We analyzed the DNA (+ strand) from wild-type ES cells before and after several I-SceI transfections (Materials and Methods). PCR products obtained with three different primer pairs were cloned and sequenced.

Figure 6A shows the DNA methylation patterns of all classes found in the mass population of ES cells: (1) Before I-

SceI cleavage (uncut); (2) recombinant GFP⁺ molecules (HR) isolated by cell sorting for HR-H or HR-L GFP expression; (3) molecules containing a rearranged I-SceI site generated by NHEJ. The methylation status of the HR molecules corresponded with the GFP expression levels of the sorted cells. Relative to the uncut parent, molecules from HR-L cells were heavily methylated, mostly in a segment of approximately 300

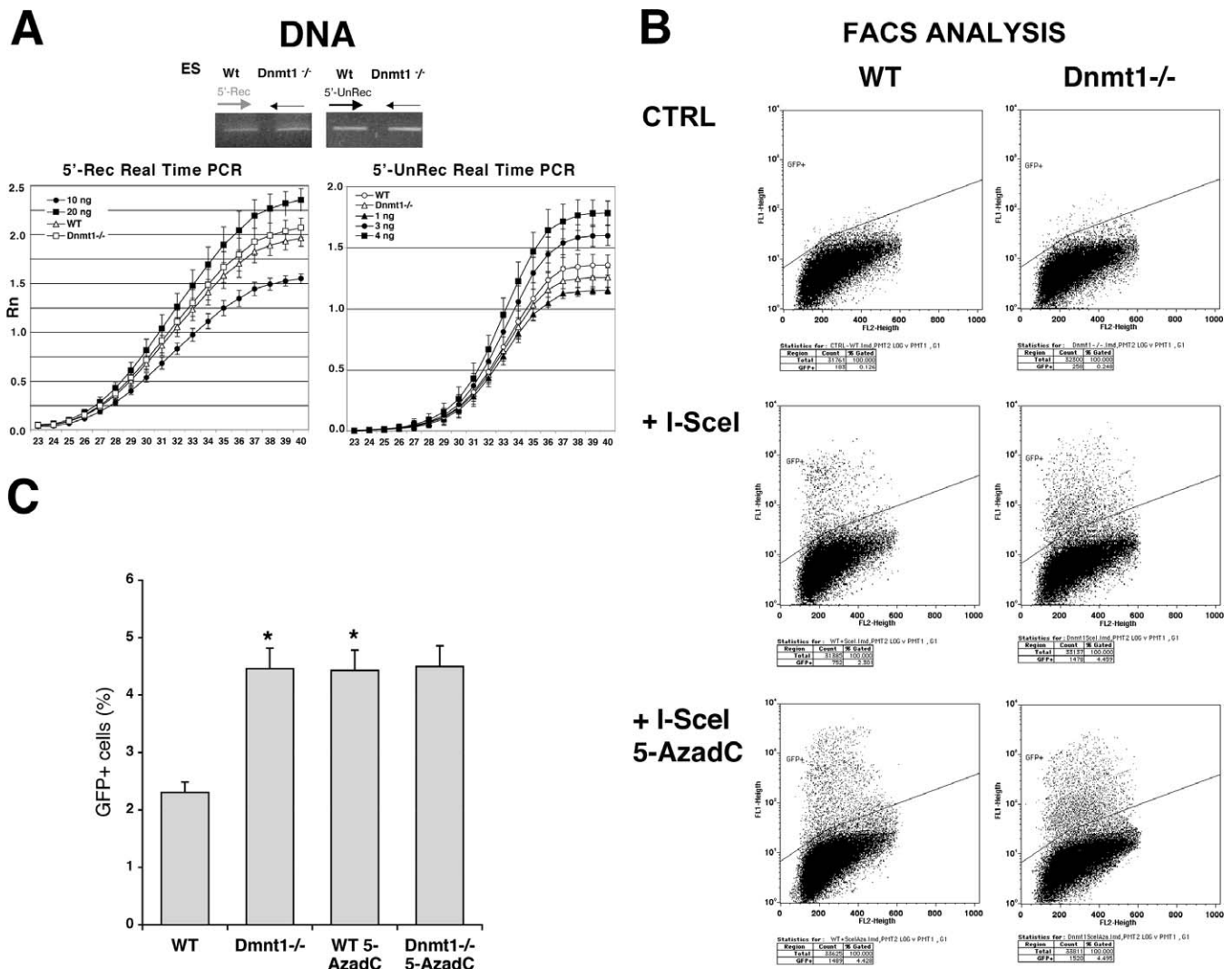


Figure 5. Dnmt1 Inhibits the Expression of Recombinant GFP Genes

Wild-type or Dnmt1^{-/-} ES cells carrying DR-GFP were transfected with the I-SceI expression vector and PSVbGal, grown 4 d, and analyzed for GFP recombination and expression.

(A) Genomic DNA from the two cell lines was PCR-amplified with nonrecombinant (5'-unrec) and recombinant (5'-rec) primers. The specificity of the products and the linearity of the reactions were controlled as described in Materials and Methods. qPCR of the same samples was carried out as described in Materials and Methods.

(B) FACS analysis of cells transfected with I-SceI is shown. The gating of GFP⁺ cells was created to exclude up to the 99.5% of wild-type untransfected ES cells. The same gating applied to Dnmt1^{-/-} cells shows a significant increase in the population expressing GFP. Following I-SceI transfection, Dnmt1^{-/-} cells were treated with 5-AzaC as described in Materials and Methods. Treatment with 5-AzaC increased the fraction of cells expressing GFP in wild-type ES but did not enhance the expression of GFP in the Dnmt1^{-/-} cells.

(C) The histogram showing the fraction of GFP⁺ cells derived from three experiments is shown. To obtain reliable values of differential GFP fluorescence in ES and Dnmt1^{-/-} cells, we compared the percentage of GFP⁺ cells, normalized for the transfection efficiency in six experiments (three in duplicate), with the Wilcoxon Kruskal-Wallis Test, *, $p < 0.012$ versus wild type.

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bp downstream to the DSB. Many of these modified CpGs represent de novo methylation sites. In contrast, molecules from HR-H cells were significantly undermethylated, both upstream and downstream to the DSB (Figures 6A and S4A). The ratio of the two classes was 1:1. Note that HR repair in this system is a short-tract strand-conversion event, since cassette II is deleted at both upstream and downstream ends. We suggest that the length of the segment showing an altered methylation pattern in the recombinants is limited by the extent of homology between cassettes I and II (400 bp downstream to the I-SceI/BclI site).

The results illustrated above suggest that Dnmt1 was

responsible for methylation at the DSB. We therefore examined molecules derived from Dnmt1^{-/-} ES cells before and after exposure to I-SceI. Recall that the Dnmt1^{-/-} mutation increased the expression level of GFP⁺ recombinants but not the recombination rate (Figure 5). As shown in Figure 6B and Figure S4B, only undermethylated recombinant molecules were generated in the mutant cells. This finding supports our contention that methylation of the recombinant molecules, shown in Figure 6A, was catalyzed by Dnmt1.

We then asked if the methylation changes following recombination in ES cells could be seen in the human Hela

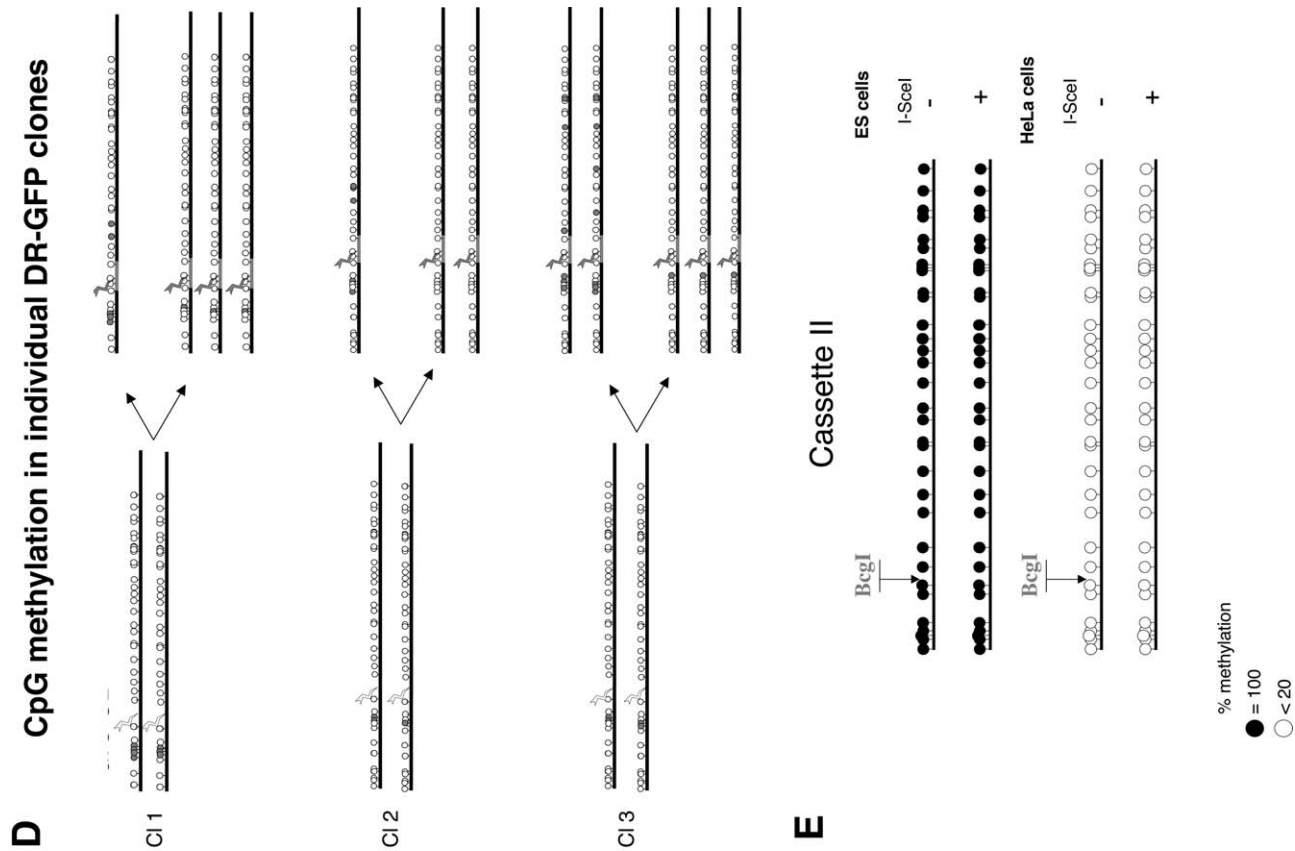
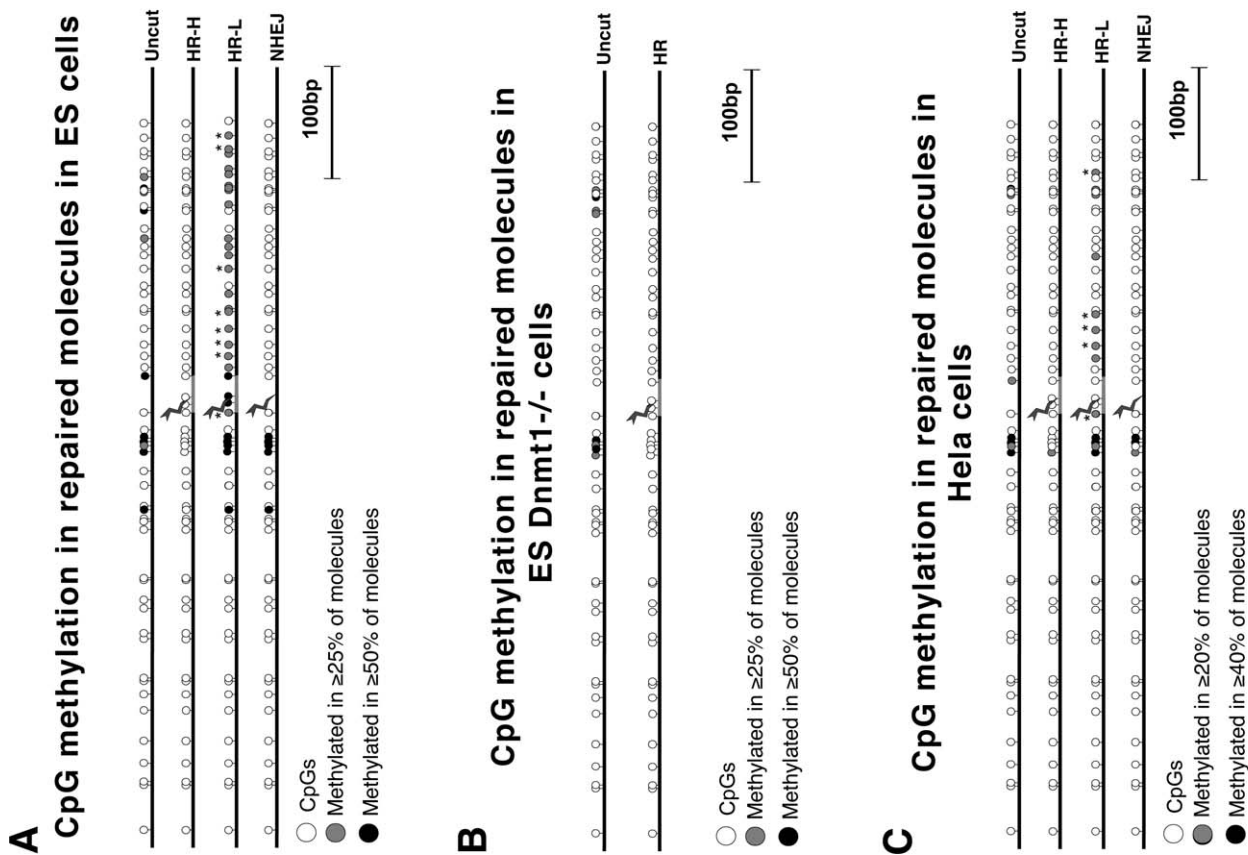


Figure 6. DNA Methylation in Repaired DNA Molecules

(A) CpG methylation in repaired molecules from ES cells is shown. DNA molecules derived from pooled ES DR-GFP cultures transfected with the I-SceI expression vector or a control plasmid were subjected to bisulfite analysis (Materials and Methods). The number of molecules in each class was as follows: (1) uncut, 40 from cells transfected with control plasmid; (2) HR-H, 25 homologous recombinant molecules from high expressor cells sorted by FACS (23) or picked randomly from mass culture; (3) HR-L, 30 recombinant molecules from low expressor cells sorted by FACS (28) or picked randomly from mass cultures; (4) molecules rearranged at the I-SceI site (NHEJ). The frequency (%) of each class was derived from several independent experiments with mass culture and fluorescent-sorted cells. HR-H 3 ± 0.5 ; HR-L 3 ± 1 ; NHEJ 2 ± 0.3 . All CpGs (white circles) flanking the I-SceI site are shown. Gray circles, CpGs methylated in $\geq 25\%$ of molecules; black circles, CpGs methylated in $\geq 50\%$ of molecules.

(B) CpG methylation in repaired molecules in ES Dnmt1 $^{-/-}$ cells is shown. DNA molecules were isolated from ES Dnmt1 $^{-/-}$ cells carrying DR-GFP, 16 from control cells and 40 from cells exposed to I-SceI. The frequency of GFP $^{+}$ cells was 5 ± 1 .

(C) CpG methylation in repaired molecules from HeLa cells is shown. DNA molecules, derived from pooled HeLa DR-GFP cultures transfected with the I-SceI expression vector or a control plasmid, were subjected to bisulfite analysis (Materials and Methods). The number of molecules in each class was as follows: (1) 25 molecules from cells transfected with control plasmid; (2) 20 recombinant molecules from low expressor cells sorted by FACS; (3) 15 recombinant molecules from high expressor cells sorted by FACS; (4) six molecules rearranged at the I-SceI site (NHEJ). The frequency (%) of each class was derived from several independent experiments with mass culture and fluorescence sorted cells. HR-H $2 + 0.5$; HR-L $2 + 1$; NHEJ $2 + 0.4$. All CpGs (white circles) flanking the I-SceI site are shown. Gray circles, molecules methylated in $\geq 20\%$ of molecules; black circles, molecules methylated in $\geq 40\%$ of molecules.

(D) CpG methylation in repaired molecules derived from individual DR-GFP clones is shown. DNA molecules were derived from clones 1, 2, and 3 of Figure 2. DNA was isolated and subjected to bisulfite analysis (Materials and Methods). Shown on the left are nonrecombinant molecules amplified with the 5'-unrec primer (see Figure 1). Shown on the right are recombinant DNA molecules isolated from cells transfected for 4 d with the I-SceI expression vector and selected for GFP expression. The arrows indicate hypermethylated DNA from clones expressing GFP at low levels and hypomethylated DNA from high GFP expressors.

(E) Methylation of GFP cassette II is not influenced by recombination. DNA methylation pattern of cassette II in HeLa DR-GFP (25 molecules) and ES DR-GFP (30 molecules) cells after transfection with I-SceI or before transfection (seven molecules) is shown. The methylation pattern of cassette II was identical in FACS sorted ES or HeLa cells.

The molecules analyzed both in ES and HeLa cells derived from at least five independent bisulfite reactions and ten independent PCRs for each group: (1) PSV β Gal transfected cells; (2) GFP $^{-}$ and (3) GFP $^{+}$ high, and (4) low expressors from I-SceI transfected cells.

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cell line. Results from the mass culture (Figure 6C) and the three individual clones (Figure 6D) are shown. Figure 6C and Figure S4C show that untreated HeLa cell DNA was relatively undermethylated compared to ES cell DNA. Nevertheless, the fraction of hypermethylated HR-L cells as well as the frequency, profile, and length of the segment containing de novo methylated CpGs in HeLa cells was similar to that observed in mouse ES cells. Recombinant molecules derived from individual clones exposed to I-SceI were likewise hypomethylated and hypermethylated in a 1:1 ratio, similar to those isolated from the pool of clones (clones 1, 2, and 3 in Figure 6D). These data shown are for the (+) strand, and were confirmed for the (−) DNA strand (unpublished data).

These results suggest that assimilation of cassette II DNA during HR induces methylation or demethylation of cassette I (see Figure 1). We wished to know if the repair process altered the methylation pattern of cassette II and if cassette II dictated the methylation of repaired cassette I. Figure 6E shows that cassette II DNA was heavily methylated in ES and undermethylated in HeLa cells in all molecules analyzed (see Figure 6E legend). Thus cassette II did not influence methylation of cassette I during recombination. Conversely, recombination with cassette I did not affect the methylation pattern of cassette II.

To get a more defined picture of the distribution of methylated CpGs in the area surrounding the I-SceI site in recombinant and parental GFP molecules, we divided the GFP segment in two regions centered on the I-SceI site: (1) a segment spanning −500 to −51 and (2) A segment at −50 to +420 relative to I-SceI site, respectively. Figure 7 shows the distribution of methylated CpGs, grouped in three classes containing 0%–1%, 1.1%–6.5%, and 6.6%–50% (0%–1%, 1.1%–3%, and 3.1%–25 % for HeLa cells) of methylated sites in these segments before or after HR. The distribution is Gaussian before I-SceI exposure in both GFP segments. After DSB and repair, only the segment located at +50 to +420, shows a bimodal distribution ($p < 0.001$) of methylated CpGs

in HeLa and ES cells. This pattern strikingly recalls the bimodal distribution in the pattern of GFP expression found following DSB-induced repair (Figures 2 and 4).

The data shown in Figures 6 and 7 summarize the statistical analysis of GFP DNA methylation before and after recombination. However, these data do not reveal the impact of recombination on the methylation pattern of individual GFP molecules. To visualize changes in individual molecules, we performed ClustalW analysis on the complete collection of GFP molecules. The difference in DNA sequence between recombinant and nonrecombinant molecules may obscure changes due to methylation. To eliminate this problem and to better assess the impact of recombination on de novo methylation, we converted the I-SceI site into a BclI restriction site in all nonrecombinant sequences and repeated the ClustalW analysis on the total pool of sequences. The molecules now are identical in sequence and differ only in methylated CpGs. ClustalW analysis of these molecules shows the methylation profiles and the degree of similarity among different molecules. Sequences containing the same methylated CpG are clustered in branches of the dendrogram (Figure 8). Figure 8A shows that nonrecombinant sequences (uncut, red) can be distinguished from recombinant HR-H (black) or recombinant HR-L (blue).

Recombination profoundly altered the methylation pattern of GFP molecules in both wild type and Dnmt1 $^{-/-}$ ES cells. Before recombination the methylation patterns of ES cells and Dnmt1 $^{-/-}$ cells are essentially identical. After recombination, two methylated populations appear in ES cells, whereas Dnmt1 $^{-/-}$ cells yield only undermethylated products (Figure 8B). Figure 8A–8C shows that recombinants can be distinguished from nonrecombinant GFP molecules by the relatedness of their methylation profiles, as shown by circled sequences. The absolute percent of methylation (indicated for each molecule) is not informative with respect to relatedness. Figure 8 indicates both the common ancestor (arrows) for each group as well as the specific classes (circles).

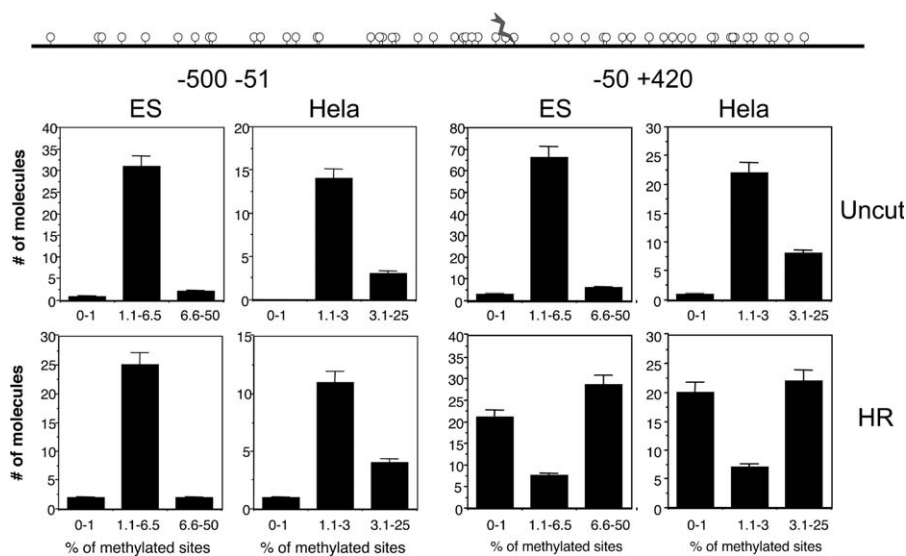


Figure 7. Distribution of Methylated CpGs before and after DSB Repair by Homologous Recombination

Statistical analysis of CpG methylation following a DSB is shown. Cassette I was arbitrarily divided in two segments located at -500 to -51 and -50 to $+420$ bp relative to the I-SceI site. Methylation was measured as percent of methylated CpGs in each molecule relative to all CpGs present in the segment. GFP molecules were arbitrarily divided in three classes: unmethylated (0%–1% methylated sites), methylated (1.1%–6.5% in ES and 1.1%–3% in Hela), and hypermethylated 6.5%–50% and 3.1%–25% in Hela). The methylated class contains all molecules between ± 1 standard deviation. After DSB and homologous recombination, the normal (Gaussian) distribution of methylated sites change to a bimodal distribution in both cell lines in the segment downstream to the break ($p < 0.001$ Shapiro-Wilk test, JMP statistical analysis software, <http://www.jmp.com>). doi:10.1371/journal.pgen.0030110.g007

In Hela cells, there are more classes, but the segregation is the same as found in ES cells (HR-L and HR-H). The circles indicating the various groups contain similar if not identical molecules (color coded), although they were derived from independent transfections and PCR analyses.

If recombination did not influence the methylation profiles of nonidentical GFP molecules, the segregation of blue (rec) and red (unrec) should be random (no circles). This appears not to be the case in either Hela or ES cells.

The simplest interpretation of the data presented in Figure 8 is that methylation is largely random in the culture but that there are preferred sites. Thus pre-existing patterns (before DSB-recombination) can be identified (arrows). After recombination, the old pattern is erased in half of the molecules (high-expressors) or significantly modified in the other half (low-expressors). Dnmt1 is essential for this modification.

Dnmt1 Is Associated with Recombinant Chromatin

Our data show that DSB repair by HR with consequent gene conversion is associated with significant methylation pattern changes in the area of the DSB. Furthermore, this methylation requires the activity of Dnmt1.

To find the molecular link between recombination and DNA methylation, we asked if Dnmt1 was associated with GFP DNA in the chromatin of cells exposed to I-SceI. Transfected Hela cells were treated with 5-AzadC and fragmented chromatin was precipitated with specific antibodies to Dnmt1. Under these conditions, incorporated 5-AzadC “freezes” Dnmt1 on the DNA and amplifies the Dnmt1 signal [18,20]. Figure 9A shows that Dnmt1 is specifically recruited to chromatin regions carrying recombined GFP DNA. Note that nonrecombinant sequences are present in large excess relative to recombined GFP DNA in input chromatin DNA. The specificity of the assay is shown by the presence of Dnmt1

on chromatin of DNA segments heavily methylated in Hela cells (the *MGMT* and *p16* genes) (Figure 9B), by the absence of the Dnmt1 signal with nonspecific antibodies (Figure 9B) and by the absence of signal with actin primers (Figure 9A, lower panel).

Methylation of the DSB Area Following Homologous Repair Is Linked to GFP Transcriptional Silencing

Our data indicate that methylation of a short segment of DNA flanking the DSB (Figure 7) is sufficient to silence GFP expression in a significant fraction of cells (HR-L) (Figures 2C, 6, and 8). Since the CMV promoter and chicken β -actin enhancer that drive GFP expression are located $\sim 1,000$ bp from the BcgII/I-SceI sites and are insulated from surrounding genomic regions (see Materials and Methods), the link between methylation and silencing is not readily evident. To explore this question, we asked if methylation inhibited transcription initiation and/or elongation. We performed RT-PCR analysis of RNA with primers derived from the upstream intron (close to the transcription initiation site), from the beginning of the GFP gene, and from the I-SceI (control cells) or BcgI (HR-L and HR-H cells) sites (Figure 10A). Since PCR reactions performed with different primers cannot be directly compared, we measured amplification of the PCR signal in a particular region of the gene by 5-AzadC. This value indicates how methylation affects transcription near the promoter and at downstream regions.

The results of Figure 10 can be summarized as follows. RNA derived from both upstream and downstream regions of the GFP gene was significantly reduced by methylation in the HR-L population. Methylation did not affect RNA synthesis in HR-H or in nonrecombinant (CTRL) clones. Finally, 5-AzadC stimulation was greater in the region of the BcgI site than further upstream (Figure 10A and 10B). Note that the

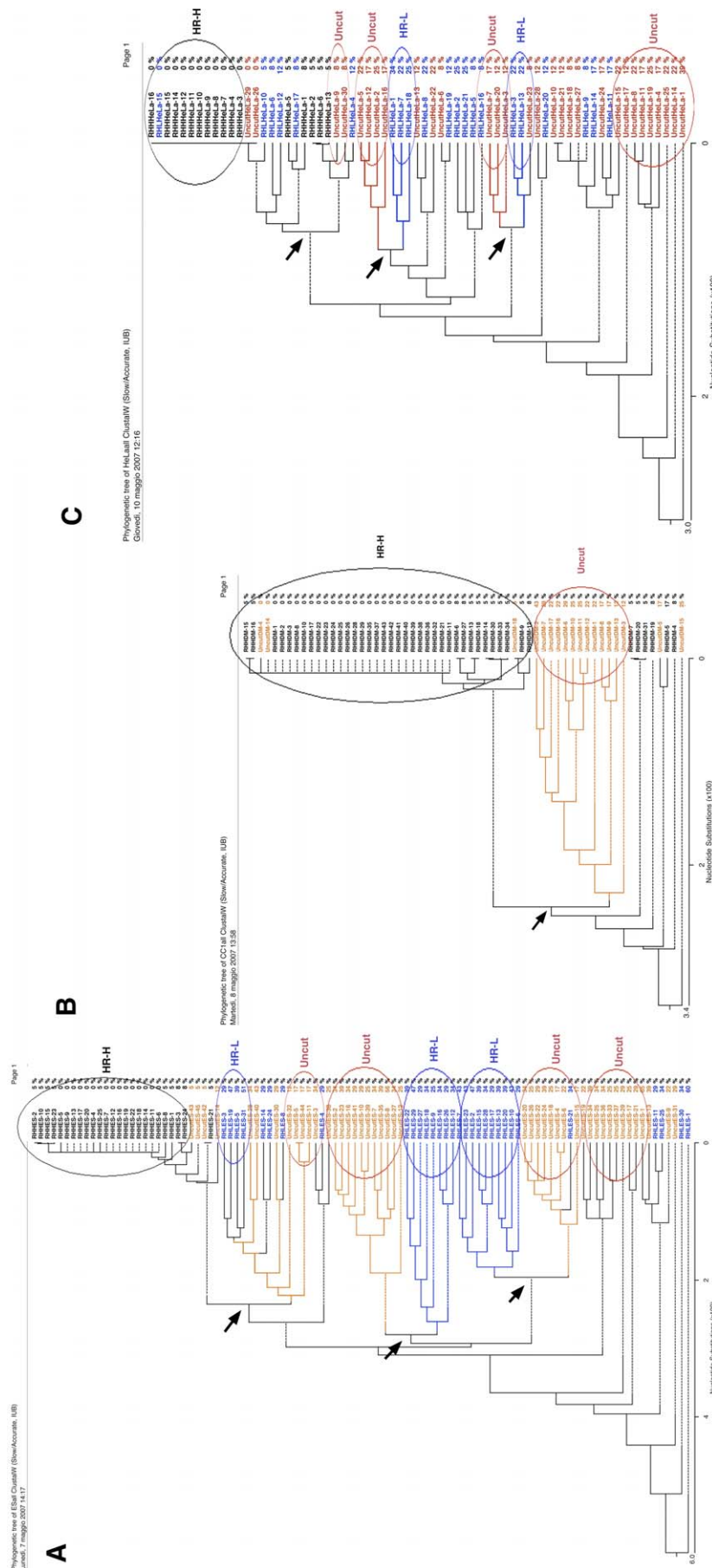


Figure 8. Methylation Patterns of Individual GFP DNA Molecules before or after HR

ClustalW analysis of individual GFP molecules (–50 to +420 bp relative to the DSB) derived from (A) ES, (B) Dnmt1^{–/–}, and (C) Hela cell lines. Recombinant molecules were derived from cells sorted by GFP immunofluorescence as HR-L and HR-H in Figure 2B and 2C. To compare only methylation patterns, the same unrecombinant molecules shown in Figure 6A–6C were artificially transformed to recombinant molecules by substituting the I-SceI with the BcgI site. The percent of sequence variation is shown on the ordinate of the phylogenetic tree (dendrogram). The three main groups indicated in the dendrogram correspond to nonrecombinant (red) and recombinant sequences (black, HR-L and blue, HR-L clones). The variation in each group depends solely on the methylation pattern. The analysis has been performed on molecules derived from ~60 independent bisulfite reactions. The sequences that showed an identical pattern of methylation from the same bisulfite analysis were eliminated. For each sequence the percent of methylation is indicated at the bottom. The sequence analysis was performed using the MegAlign 7.0.1 a module of the Lasergene-DNASTAR software.

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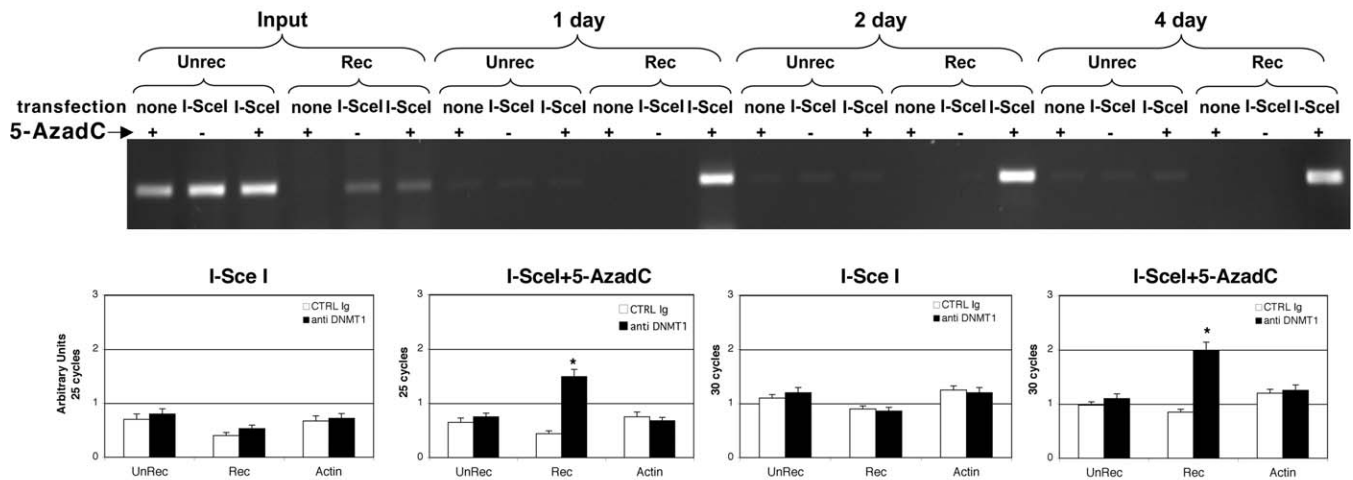
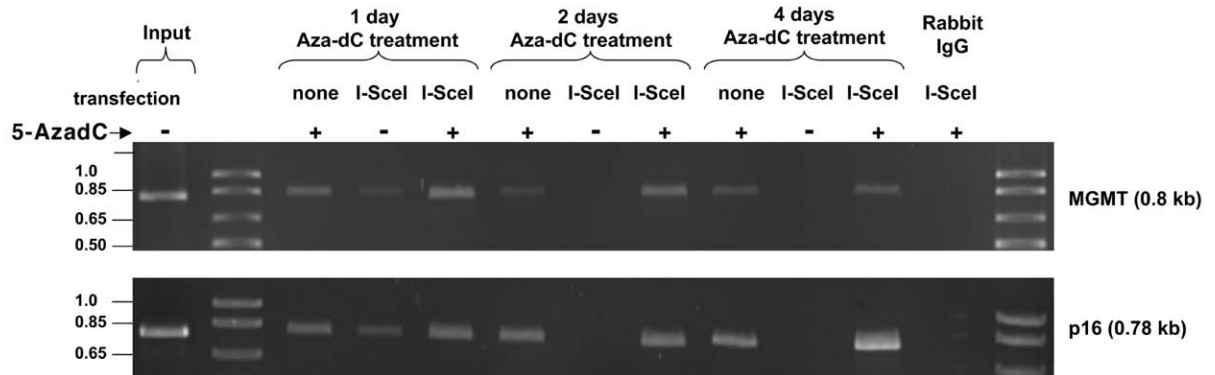
A**B**

Figure 9. Dnmt1 Selectively Binds Recombinant GFP Chromatin

Hela cells carrying DR-GFP were transfected with I-SceI and treated 24 h later with 1 μ M 5-Aza-dC for 1, 2, and 4 d. Chromatin immunoprecipitation (ChIP) was carried out as described in Materials and Methods.

(A) PCR of immunoprecipitated DNA with antibodies to Dnmt1 is shown. None indicates chromatin derived from cells transfected with control plasmid, (–) or (+) indicates the treatment with 5-Aza-dC. Rec, unrec indicate the primers used for amplification. The lower panel shows the statistical analysis of PCR reactions carried out at 25 and 30 cycles when the reactions with the three sets of primers were in the linear range. Immunoprecipitations were carried out with nonspecific immunoglobulin G (Control immunoglobulin G) and anti-Dnmt1 specific antibodies. The primers used were: (1) unrec; (2) rec; and (3) actin (*, $p < 0.01$ versus control immunoglobulin G).

(B) The conditions are the same as (A). MGMT and p16 indicate the primers used for amplification.

doi:10.1371/journal.pgen.0030110.g009

difference between 5' and 3' end transcript levels may be artificially amplified by the fact that only the 3' end primers are selective for recombinant RNA (see Figure 1). Since sorted cells may contain copies of unrecombined DR-GFP, these units can generate nonrecombinant transcripts, which are not stimulated by 5-Aza-dC (see CTRL). As a result, the differential (– or + 5-Aza-dC) levels of 5' end may appear lower than the 3' end transcripts. Notwithstanding this limitation, we find a significant and reproducible stimulation of 5' end transcript by 5-Aza-dC ($p < 0.01$).

Our data indicate that CMV promoter activity is inhibited by methylation at the DSB and suggest further that elongation may also be hindered by methylation of the repaired segment. We propose that this inhibition is

triggered by changes in the chromatin domain that includes the repaired DSB. Nucleosome structure is known to affect both transcription initiation and elongation [21].

Discussion

Homology-Directed Repair and DNA Methylation

Here, we demonstrated a causal relation between HR, DNA methylation, and gene silencing. PCR and fluorescence-based recombination assays were used to monitor HR following introduction of a DSB with I-SceI at a selected genomic site. The recombination system was that of Jasin et al. [17] in which direct repeats of defective GFP genes recombine to yield a wild-type product by gene conversion. We found both hypermethylated and hypomethylated recombinant DNA

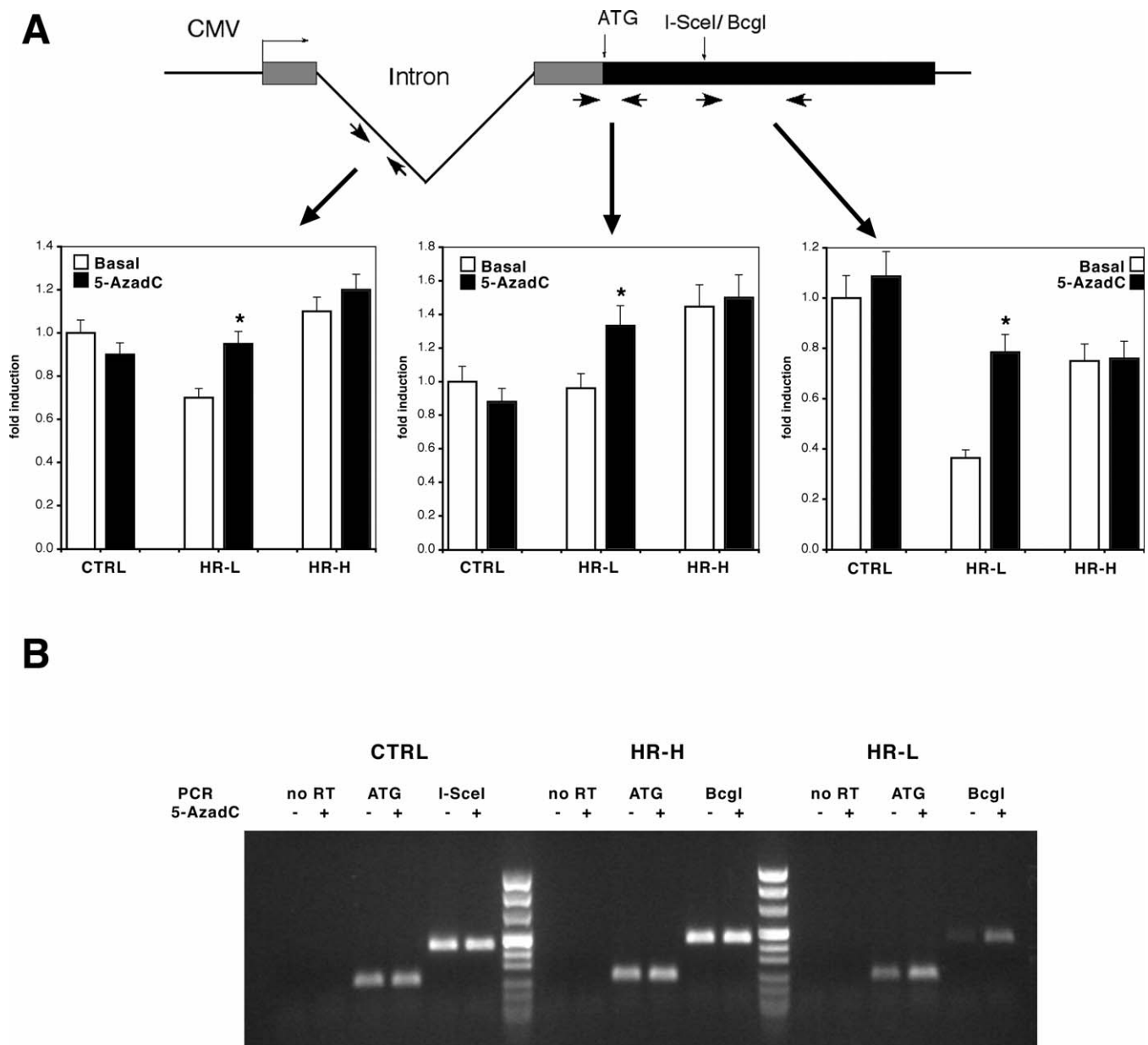


Figure 10. Mapping of GFP Transcription in Recombinant and Nonrecombinant Cells with and without 5-AzadC Treatment

(A) A schematic of the DR-GFP transcriptional unit shows the location of the CMV promoter, intron, and GFP coding sequence. Primers used for quantitative RT-PCR and RT-PCR are indicated by arrows. Different sets of primers were derived from the intron (738–757, forward 5'-CGTTACTCCACAGGTGAGC-3'; 966–948, reverse 5'-CGCCCGTAGCGCTCACAGC-3'), AUG (1,666–1,685, forward 5'-TACAGCTCCTGGGCAACGTG-3'; 1,911–1,892, reverse, 5'-TCCTGCTCCTGGGCTTCTCG-3'), and BclI/I-SceI (described in Figure 1A) segments of GFP gene. Control (DR-GFP cells transfected with pBluescript), HR-L, and HR-H cells were treated with 40 μ M 5-AzadC for 48 h. Total RNA was extracted 24 h later and subjected to quantitative RT-PCR with the primers indicated. The data, derived from three independent cDNAs, are shown as fold induction by 5-AzadC over the basal control, normalized to GAPDH and β -actin. The primers used to amplify the control samples were those indicated as I-SceI unrec (Figure 1A).

(B) Shown is RT-PCR with the same cDNAs indicated in (A) at 30 cycles.
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products in about a 1:1 ratio. The hypermethylated recombinants expressed low levels of GFP compared to the hypomethylated recombinants. Silencing of these GFP genes was due to DNA methylation and was reversed by 5-AzadC treatment after exposure to I-SceI. Demethylation prior to DSB generation was ineffective. Similarly, murine ES cells lacking *Dnmt1*^{-/-} yielded more clones expressing high levels of GFP than wild-type controls.

Silencing of HR GFP differs from transgene silencing observed by others [19]. Expression of a transfected wild-type GFP was only slightly reduced over a three-week period, whereas HR-L clones were silenced during the two weeks following exposure to I-SceI (Figure 2B). Likewise, we saw no silencing of uncleaved or wild-type GFP genes, since their expression was not stimulated by 5-AzadC (Figures 2 and 10).

Single clones did not display the same methylation pattern

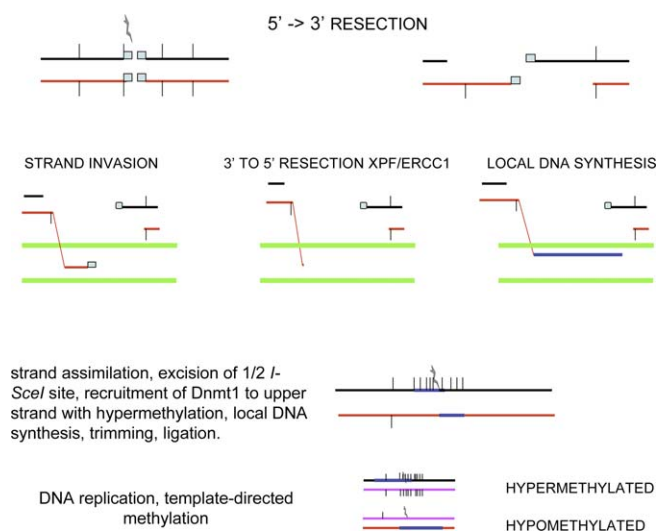


Figure 11. HR DNA Molecules Are Marked by Methylation Pattern Changes

A drawing illustrates how HR affects methylation. The case shown is a gene conversion event following a DSB at a specific chromosomal site with the GFP gene. The invasion of GFP cassette II (green) maintains the linearity of the DNA molecule at the I-SceI site. Methylation of only one repaired strand (the leading strand, in this specific example) is followed by an intermediate hemimethylated DNA molecule. Replication of this molecule yields fully methylated and nonmethylated DNA molecules. We suggest that Dnmt1 and the DSB repair machinery (BASC, BRCA1-associated surveillance complex) are strand-selective. doi:10.1371/journal.pgen.0030110.g011

following I-SceI exposure. Since the I-SceI vector persisted for about one week, cells were subjected to repeated cycles of cleavage and repair, as shown by phosphorylated- γ -H2AX foci (Figure S5). The resultant methylation pattern varied from cell to cell, although preferred modification sites were seen (Figure 8).

The methylation patterns generated by gene conversion are not seen in the parental DNA. Molecules unexposed to I-SceI had an average methylation density of 1%–3% (Figure 7). About half of the converted molecules were hypermethylated (7%–50% of methylated sites in the 200 bases flanking the I-SceI site). The remainder were hypomethylated (0%–1% in these 200 bases).

To illustrate the two aspects of DNA methylation, our data on the methylation status of GFP molecules are presented either as percent of methylated CpGs in different GFP segments (Figures 6 and 7) or as profiles of methylation, showing which specific CpGs are methylated and the degree of similarity among different molecules (Figure 8). The analysis of single molecules is highly informative, allowing us to identify common and unique methylation profiles between recombinant and nonrecombinant molecules. HR, which occurs stochastically on the nonrecombinant GFP molecules, erases or induces methylation at the DSB and generates the bimodal methylation patterns shown in Figures 7 and 8.

Hypomethylated and hypermethylated molecules in ~1:1 ratio were also found in recombinants derived from single clones, indicating that hypomethylated or hypermethylated GFP sequences did not segregate in individual clones. Thus, both HR-L and HR-H recombinants are generated in both mass cultures or in clones containing a single integration site

(Figures 2 and 4). Note that the GFP expression levels of wild-type GFP transfectants show a Gaussian distribution, totally unlike the bimodal distribution observed after recombination. This finding implies that DNA repair generates one hypermethylated and one hypomethylated strand and replication yields double-stranded molecules that retain the methylation pattern of the precursor strand (Figures 8 and Figure 11).

GFP⁺ cells are generated from short-tract gene conversion of the I-SceI site on GFP cassette I to the BclI site on GFP cassette II. Cassette II, which provides the template strand, has only 395 bp of homology downstream to the I-SceI of cassette I. This may explain why the altered methylation pattern in the recombinant molecules is limited to a small region flanking the DSB.

The methylation status of cassette II did not dictate the modification of the recombined GFP gene. Cassette II was hypermethylated in ES and hypomethylated in HeLa cells, whereas the methylation patterns of the repaired DNA of cassette I were similar in both cell types. Furthermore, HR of cassette I did not change the methylation pattern of cassette II.

A Model Explaining the Methylation Pattern of the Repaired DNA

DNA cleaved at the DSB in cassette I is filled in with newly synthesized DNA templated by cassette II, with gene conversion at the I-SceI site, or by a sister chromatid, without gene conversion. We propose that only one of the newly synthesized strands is methylated by Dnmt1, which is recruited to the DSB by proliferating cell nuclear antigen [11] and marks the HR molecules (Figure 8). Replication of the hemimethylated intermediate yields hypermethylated and hypomethylated DNA products in a 1:1 ratio. In the case of gene conversion, the length of the hyper- or hypomethylated track does not exceed the length of the cassette II template (Figure 11).

We find that Dnmt1 can act as a *de novo* methyltransferase, i.e., that it can recognize unmethylated DNA as a substrate. Although it is commonly thought that Dnmt1 cannot promote *de novo* methylation, in fact the preference of Dnmt1 for hemimethylated versus unmethylated DNA is only 7- to 21-fold [22]. The possibility that the known *de novo* methyltransferases, Dnmt3a and Dnmt3b act at the DSB seems unlikely. First, we find that Dnmt1^{−/−} ES cells show a greater frequency of GFP⁺ recombinants than wild-type cells. Second, these methyltransferases are not recruited to sites of DNA damage [12]. Third, all GFP recombinants were unmethylated in Dnmt1^{−/−} ES cells, although the parental molecules were normally methylated (Figure 8B). Fourth, Dnmt1 was specifically associated with HR GFP molecules (Figure 9). The similarity in the methylation profiles of parental DNA molecules in Dnmt1^{−/−} cells to wild-type cells implies that Dnmt3a and Dnmt3b can partially substitute for Dnmt1 during normal replication, but cannot replace Dnmt1 in methylation induced by HR (Figure 8A and 8B).

Repair and Gene Silencing

Alteration of the methylation pattern following gene conversion was restricted to 200–300 bp flanking the DSB. Changes in methylation did not extend to the CMV promoter that drives GFP expression or to cassette II. How can this

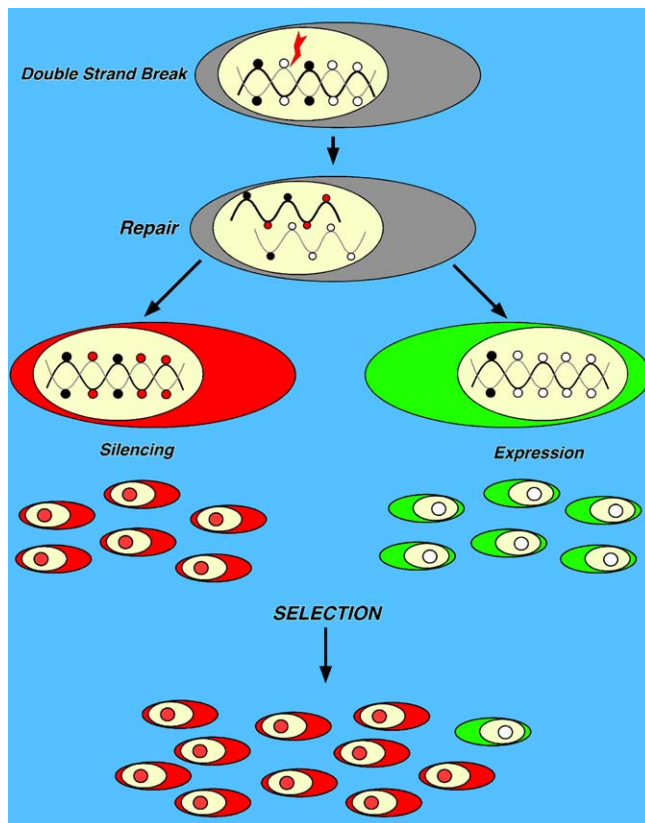


Figure 12. Biological Consequences of Recombination-Induced Methylation Switch

A drawing illustrates the sequence of events leading to silencing or expression of HR DNA segments. Red circles represent de novo methylated CpGs induced by HR. Black circles represent methylated CpGs before HR. Since silencing depends on the location of de novo methylated CpGs and DNA damage is random, HR-induced methylation is also random. If the expression of the repaired gene is harmful, only cells inheriting the silenced copy will survive. Conversely, if the function of the repaired gene is beneficial, cells inheriting the undermethylated copy will have a selective advantage.
doi:10.1371/journal.pgen.0030110.g012

restricted methylation induce silencing of the recombinant GFP gene? The data shown in Figure 10 indicate that methylation at the BcgI site downregulates transcription initiation and probably partially blocks transcription elongation. We propose that local methylation initiates chromatin compaction and, ultimately, silencing of genes near the DSB [23]. Since the GFP gene is flanked by a puromycin-resistance gene transcribed on the other strand, which remains active, and is driven by a strong and insulated CMV promoter, the mechanism of inhibition appears to be selective. The nature and the extent of propagation of the silencing signal remains to be explored.

Loss of Heterozygosity and DNA Damage Repair

Our data indicate that HR leading to gene conversion is associated with de novo methylation of short DNA segments. We wondered if this association extended to other examples of DNA repair. We examined the methylation patterns in DNA that had undergone loss of heterozygosity (LOH), which in yeast is associated with DNA damage repair, possibly via gene conversion [24].

Assuming that LOH in tumors might also derive from HR repair and gene conversion, we analyzed *PTPDJ*, a gene that is frequently subject to LOH and silencing in thyroid tumors [25]. DNA from three independent tumors (M39, M43, and M107) carried a cluster of methylated CpGs in the *PTPDJ* promoter region. This DNA displayed LOH on one or both sides of the cluster. Tumor DNA without LOH, or DNA extracted from three normal thyroids, did not contain this methylated cluster. In addition 50% of the molecules in the LOH population were unmethylated in the CpG region (Figure S6). We found that 90% of the DNA molecules in tumor M43 showed LOH and were therefore recombination products. The similarity between the methylation patterns of M43 and GFP recombinants suggests that LOH may reflect DSB repair and associated methylation. Note that LOH in these tumors is not due to chromosome loss. The *PTPDJ* copy number was equivalent in normal and tumor tissues (unpublished data).

Although the LOH markers were at least 21 kb from the region analyzed (see Figure S6 legend), the methylated region was clustered in the same area in these three independent tumors. It is possible that this region is close to a recombination hot spot or a replication origin, i.e., a site of DSB formation, HR, and gene conversion.

In conclusion, these results argue for a cause-effect relation between HR repair and de novo DNA methylation. Discontinuous LOH is a signature of oxidative DNA damage and closely resembles a mutational pattern found in human tumors [26]. Hypermethylation of short DNA tracts in tumors or in aging represents the consequence of gene conversion. We note that silencing of tumor suppressor genes by methylation is characteristic of tumor cells [27]. Indeed, evidence of enhanced DSB formation in hyperplastic precancerous cells precedes the genomic instability and loss of p53 characteristic of more advanced tumors [28,29]. Selection of methylated silenced alleles will eliminate unmethylated ones from the population (Figure 12).

Our data also imply that gene imprinting may be linked to homologous recombination events. In this perspective it is noteworthy that a systematic genome-wide analysis reveals that human imprinted chromosomal regions are historical hot spots of recombination [30].

Materials and Methods

Cell culture. HeLa-DR-GFP stable lines were cultured in RPMI medium supplemented with 10% fetal bovine serum (Invitrogen, <http://www.invitrogen.com>), 1% penicillin-streptomycin, and 2 mM glutamine. ES-DR-GFP wild-type and ES stable lines were cultured in DMEM medium supplemented with 15% fetal bovine serum (Invitrogen), 50 U of penicillin-streptomycin/ml, 0.1 mM β -mercaptoethanol, 0.1 mM nonessential amino acids (Invitrogen), and 500 U/ml of leukemia inhibitory factor (Invitrogen). Cells were grown on gelatine-coated dishes without feeder cells and cultured for a maximum of 20 generations [31]. The two cell lines were grown at 37 °C in 5% CO₂.

Stable and transient transfections. DR-GFP plasmid was 14,735 bp containing: CMV IE enhancer (1–385); chicken beta-actin promoter (386–751); chicken beta-actin first intron (752–1,622); rabbit beta-globin second intron (1,623–1,670); rabbit beta-globin third exon (1,671–1,724); EGFP with a STOP (16) codon at I-SceI site (1,740–2,756, I-SceI at 2,135); SV40 splice/polyadenylation signal (2,757–3,023); polyadenylation signal from phosphoglycerate kinase gene (3,025–3,607) for the puromycin resistance gene (3,600–4,200); a truncated EGFP gene sequence (5,609–6,138); and 6,450 bp of mouse genome ([17] and A. Porcellini, unpublished data). HeLa-DR-GFP stable lines were transfected with lipofectamine as recommended by

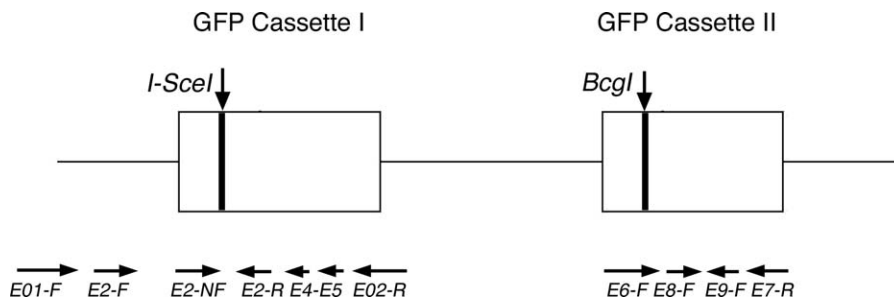


Figure 13. Location of the Primers Used for Methylation Analysis

Schematic of the DR-GFP transcriptional unit is shown. The arrows indicate the specific primers.

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the manufacturer (Invitrogen) with 2 μ g of circular pDR-GFP plasmid and selected in the presence of puromycin (2 mg/ml). Puromycin-resistant colonies (~ 200 clones) were seeded at 3×10^5 cells per 60-mm plate and transfected with 2.5 μ g pC β ASce plasmid DNA on the following day by lipofectamine transfection (Invitrogen). Cells were harvested 2 or 4 d after transfection. Wild-type ES and Dnmt1 $^{-/-}$ lines [5] were transfected by electroporation in tissue culture medium with 50 μ g circular pDR-GFP plasmid and plated in the presence of puromycin at 0.5 mg/ml. Puromycin-resistant colonies were transfected by electroporation with 10 μ g pC β ASce. At 24 h posttransfection, cells were expanded for a week in selective medium, aliquoted, and frozen at 135 $^{\circ}$ C. Aliquots of transiently transfected cells were cultured for 2 wk and analyzed. Pools of clones (ES wild type, Dnmt1 $^{-/-}$, or Hela) were generated in two or three independent transfections and frozen in aliquots. Transient transfections with I-SceI were carried at different times of culture after the primary transfection (for the isolation of single clones, we used cells cultured for more than 1 mo). We used the same conditions of growth ($\sim 40\%$ confluency starting from freshly frozen aliquots). Transfection efficiency was measured by assaying β -galactosidase activity of an included pSV β Gal vector (Promega, <http://www.promega.com>). Normalization by FACS was performed using antibodies to β -gal or pCMV-DsRed-Express (Clontech, <http://www.clontech.com>). pEGFP (Clontech) was used as GFP control vector.

Drug treatments. Hela-DR-GFP cells were transfected with control vector or pC β ASce plasmid. The cells were plated at low confluence 24 h later and incubated with 5-AzaC (5 μ M) for 48 h. The cells were analyzed 48 h later. When the drug was used before transfection, the treatment was terminated 48 h before transfection. The analysis of the cells was carried out 5 d after transfection. Drug treatments were performed under the same growth conditions (40% confluency from freshly thawed aliquots).

RNA and DNA extraction. Total RNA was extracted using Triazol (Gibco/Invitrogen). Genomic DNA extraction was performed with following protocol: cellular pellet was resuspended in 10 mM TRIS (pH 7.8) and 50 mM NaCl solution. After addition of 1% SDS the sample was gently mixed. Proteinase K, at a final concentration of 20 μ g/ml, was added and the mixture was incubated at 55 $^{\circ}$ C overnight. The following day, hot 1.5 M NaCl (70 $^{\circ}$ C) was added to the mixture, and the DNA was extracted by phenol/chloroform. DNA was ethanol precipitated, dried, and resuspended in TE buffer.

qRT-PCR, qPCR, RT-PCR, and PCR. cDNA was synthesized in a 20- μ l reaction volume containing 2 μ g of total RNA, four units of Omniscript Reverse Transcriptase (Qiagen, <http://www.qiagen.com>), and 1 μ l random hexamer (20 ng/ μ l) (Invitrogen). mRNA was reverse-transcribed for 1 h at 37 $^{\circ}$ C, and the reaction was heat inactivated for 10 min at 70 $^{\circ}$ C. The products were stored at -20° C until use. PCR was performed in a 50- μ l reaction mixture containing 2 μ l of synthesized cDNA product or 0.5 μ g of genomic DNA, 5 μ l of 10 \times PCR buffer, 1.5 mM MgCl₂, 0.3 mM dNTP, 1.25 unit of Taq polymerase (Roche, <http://www.roche.com>), and 6 μ M of each primer. The primer sequences that were used for the different mRNAs or DNAs were: unrec 5'-GCTAGGGATAACAGGGTAAT-3'; rec 5'-GAGGCGGAGGGCGATGCC-3'; reverse oligo 5'-TGCACGCTGCCGTCCTCG-3' (443-bp amplified fragment); β -actin/R 5'-AAAGCCATGCCAATCTCATC-3'; β -actin/L 5'-GATCATTGCTCCTCCTGAGC-3' (250 bp amplified fragment); GAPDH forward oligonucleotide 5'-TTCACCAACCACCATGGAGAAGGCT-3'; reverse 5'-ACAGCCTTGGCAGCACCAGT-3' (346-bp amplified frag-

ment). Amplifications were performed in a Primus thermocycler (MWG/Biotech, <http://www.mwg-biotech.com>) using the following program: 95 $^{\circ}$ C /5 min \times 1 cycle; 95 $^{\circ}$ C /45 s, 57 $^{\circ}$ C /30 s, and 72 $^{\circ}$ C /2 min \times 28–35 cycles; 72 $^{\circ}$ C /10 min \times 1 cycle. The number of cycles were selected and validated by running several control reactions and determining the linear range of the reaction. A total of 15 μ l of the PCR products were applied to a 1.2% agarose gel and visualized by ethidium bromide staining. Densitometric analysis was performed using a phosphorimager. Each point was determined for at least three independent reactions.

Quantitative (q)RT-PCR and qPCR were performed three times in six replicates on a 7500 RT-PCR System (Applied Biosystems, <http://www.appliedbiosystems.com>) using the SYBR Green-detection system using the following program: 95 $^{\circ}$ C /5 min \times 1 cycle, 95 $^{\circ}$ C /45 sec and 62 $^{\circ}$ C /45 min \times 40 cycles. Reference curves were generated for 1, 3, 5, 10, and 1,000 ng of DR-GFP plasmid in six replicates for copy number assay and from 0.1, 0.5, 1.0, and 5.0 μ l of each cDNA for GAPDH in the relative assay.

BclI digestion of DNA or cDNA prior to PCR. cDNA prepared from 2 μ g of total RNA extracted from Hela-DR-GFP transfected with pC β ASce or control plasmid was digested with BclI restriction enzyme as recommended by the manufacturer (New England Biolabs, <http://www.neb.com>). We used 2.5 μ l of cleavage reaction to perform PCR with primers that specifically amplify unrecombinant (unrec) and recombinant (rec) GFP sequence. For the FACS analysis, Hela-DR-GFP and ES-DR-GFP cells were transfected with pC β ASce or control plasmid and after 2 or 4 d were treated with trypsin and collected. After two washes with PBS, cells were resuspended in 500 μ l of PBS 1 \times . A total of 6×10^4 cells were analyzed.

Bisulfite DNA preparation, PCR, and sequence analysis. Sodium bisulfite analysis was carried out essentially as described by Frommer et al. [32]. A total of 8 μ g sample of total DNA was digested with EcoRI restriction enzyme (New England Biolabs) and denatured in 0.3M NaOH for 15 min at 37 $^{\circ}$ C in a volume of 100 μ l. We then added 60 μ l of 10 mM hydroquinone and 1.04 ml of 3.6 M NaHS₃ (pH 5). Reaction mixtures were incubated at 50 $^{\circ}$ C for 16 h in the dark. DNA was desalted and concentrated using GeneClean (Qbiogene/Bio101, <http://www.qbiogene.com/products/bio101.shtml>), denatured with 0.3M NaOH for 15 min at 37 $^{\circ}$ C, neutralized with 3M NH₄OAc (pH 7), and ethanol precipitated. An aliquot of DNA was amplified with the following modified primers (Figure 13): primers: E01-F, 5'-GTGTGATTGGTGGTTTTAGAGT-3'; E02-R, 5'-CCATCCTCAA-TATTATAACAAT-3'; E2-F, 5'-GGAGTTGT-TATTGGGGTGGTGTGTTTATTTTGGT-3'; E2-NF, 5'-TGGATGGTGTATGTAATGGTTATAAGTTT-3'; E2-R, 5'-GTTGTGTTTATAGGATGTTGTTG-3'; E4-R, 5'-ACTTATACAACT-CATCCATACCAAAAATAATCC-3'; E5-R, 5'-ACTTATACAACT-CATCCATACCGAAAATAATCC-3'; E6-F, 5'-GGT TGT TATGAATAAAGGTGGTTATAAGA-3'; E7-R, 5'-CTCACTCAT-TAAACACCCCAAACTTTACAC-3'; E8-F, 5'-GAAGATTTTTPy-GATTTGT AGTTTAAGTTTATAGG-3'; and E9-R, 5'-GAAGATTTTTPyGATTTGTAGTTTAAGTTTATAGG-3'.

All PCR reactions were carried out in 100- μ l reaction mixtures containing 5 μ l bisulfite-treated genomic DNA, 200 μ M dNTPs, 10 pmol of each primer, 1 mM MgCl₂, 50 mM KCl, 10 mM Tris, 5% dimethyl sulfoxide, and 2 U of Taq polymerase (Stratagene, <http://www.stratagene.com>) in a Primus thermocycler (MWG/Biotech) under the following conditions: 95 $^{\circ}$ C /5 min and 70 $^{\circ}$ C /2 min \times 1 cycle, 97 $^{\circ}$ C /1 min, 54 $^{\circ}$ C /2 min, and 72 $^{\circ}$ C /1 min \times 5 cycles; 95 $^{\circ}$ C /0.5 min, 52 $^{\circ}$ C /2 min, and 72 $^{\circ}$ C /1 min \times 25 cycles; and 72 $^{\circ}$ C /3 min \times 1 cycle. We used 2

μl of the first PCR to perform the second PCR (PCR nested or seminested) under the same PCR conditions. PCR products were cloned into pGEM-T Easy Vector (Promega), and at least 20 independent clones for each fragment were sequenced with the T7 primer.

Sequence analysis and alignment were performed using MegAlign software (a module of the Lasergene Software Suite for sequence analysis by DNASTAR, <http://www.dnastar.com>) for MacOSX. Statistical analysis was performed using the JMP 6.0.3 statistical analysis software by S.A.S (<http://www.sas.com>).

LOH and thyroid tumors. Samples and procedures for the analysis of LOH in normal and tumor DNAs were carried out as described in Liu et al. [25].

Chromatin immunoprecipitation. We seeded 7×10^5 cells per well of HeLa-DR-GFP in a six-well plate one day before transfection. Cells were transfected with 2 μg of I-SceI plasmids using Lipofectamine 2000 (Invitrogen). Cells were plated in a 150-mm plate 24 h later and incubated with 1 μM of 5-AzadC (Sigma, <http://www.sigmaaldrich.com>) for 24 h, 48 h, or 96 h. The medium and 5-AzadC were changed every 24 h. Cells were harvested and GFP positive cells were scored by FACS. Remaining cells were stored at -80°C .

ChIP-IT Express kit (Active Motif, <http://www.activemotif.com>) was used for ChIP experiments. Because cells were treated with 5-AzadC, formaldehyde cross-linking and reverse cross-linking steps were omitted.

Chromatin isolation and enzymatic shearing. A total of $\sim 1 \times 10^7$ cells were resuspended in 0.5-ml ice-cold lysis buffer supplemented with 2.5 μl protease inhibitor cocktail (ChIP-IT Express kit, Active Motif) and 2.5 μl of 100 mM PMSF. After 30 min incubation on ice, cells were homogenized with a Dounce homogenizer (ten strokes), and nuclei were collected by centrifugation at 2,000 g/5 min. The nuclei were resuspended in 0.5 ml of digestion buffer (provided by the kit) and incubated at 37°C for 10 min with 25 μl of enzymatic shearing cocktail (200 U/ml, ChIP-IT Express kit, Active Motif). The reaction was stopped by adding 10 μl of 0.5 M EDTA. The sheared chromatin was stored at -80°C . An aliquot of sheared chromatin was further treated with proteinase K, phenol/chloroform extracted, and precipitated to determine DNA concentration and shearing efficiency (input DNA).

Capture of chromatin on magnetic beads. The ChIP reaction was set up according to the manufacturer's instructions. Briefly, the sheared chromatin (corresponding to 18 μg of DNA) was mixed with protein G magnetic beads and 2 μl of anti-Dnmt1 antibody (New England Biolabs) or 0.8 μg of normal rabbit IgG (Santa Cruz, <http://www.scbt.com>). The reaction mixture was incubated at 4°C for 48 h. Beads were washed with wash buffer and immunoprecipitated DNA was recovered.

PCR analysis for unrec and rec. PCR for rec was performed in a 20-μl reaction mixture containing 5 μl of recovered DNA, 0.2 mM dNTP, 1.25 units of HotStarTaq polymerase (Qiagen), and 5 μM of each primer. Amplifications were performed using following conditions: $95^\circ\text{C}/15 \text{ min} \times 1 \text{ cycle}$; $95^\circ\text{C}/45 \text{ sec}$, $65^\circ\text{C}/30 \text{ sec}$, and $72^\circ\text{C}/1 \text{ min} \times 40 \text{ cycles}$; and $72^\circ\text{C}/10 \text{ min} \times 1 \text{ cycle}$.

PCR for unrec was performed in a 20-μl-reaction mixture containing 1 μl of recovered DNA. Amplifications were performed using following conditions: $95^\circ\text{C}/15 \text{ min} \times 1 \text{ cycle}$; $95^\circ\text{C}/45 \text{ sec}$, $52^\circ\text{C}/30 \text{ sec}$, and $72^\circ\text{C}/1 \text{ min} \times 30 \text{ cycles}$; and $72^\circ\text{C}/10 \text{ min} \times 1 \text{ cycle}$.

PCR for Input was performed in a 20-μl-reaction mixture containing 100 ng of input DNA. Amplifications were performed with 30 cycles.

PCR analysis for positive and negative controls. To validate the Dnmt1 ChIP reactions, the following primers were used for PCR: hMGMT, 813 bp, (−726 to +87); MGMT-F, GAGTCAGGCTCTGG-CAGTGT; MGMT-R, GAGCTCCGCACTCTTCCGG; pP16, 778 bp, (−656 to +132); p16-F, GCAGTCCGACTCTCCAAAAG; p16-R, AGC-CAGTCAGCCGAAGGC; β-actin, 250 bp; β-actin/R, AAGCCATGC-CAATCTCATC; and β-actin/L, GATCATGTGCTCCTCTGAGC.

PCR was performed in a 20-μl reaction mixture containing 5 μl (1 μl for actin) of recovered DNA, 0.2 mM dNTP, 1.25 unit of HotStarTaq polymerase (Qiagen), and 5 μM of each primer. Amplifications were performed using following conditions: $95^\circ\text{C}/15 \text{ min} \times 1 \text{ cycle}$; $95^\circ\text{C}/45 \text{ sec}$, $50^\circ\text{C}/30 \text{ sec}$, and $72^\circ\text{C}/1 \text{ min} \times 40 \text{ cycles}$; and $72^\circ\text{C}/10 \text{ min} \times 1 \text{ cycle}$.

Supporting Information

Figure S1. DNA and RNA Analysis of HR GFP

(A) PCR of genomic DNA was performed at different cycles (25 and 30) with a reference marker included (actin). In all experiments, PCRs

were carried out at different DNA concentrations and cycles to determine the linear range of the reaction. This is illustrated in the graph, where the PCR product (ordinate) is plotted versus the number of PCR cycles.

(B) RNA analysis was performed on double-stranded cDNA synthesized from total RNA. RT-PCR analyses were carried out in the linear range of the reaction. To confirm that the PCR products represented recombined and unrecombined DNA, the cDNA was treated with BclI before amplification (quality check). Only recombinant GFP cDNA is cleaved by BclI (see Materials and Methods).

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Figure S2. DNA and RNA Analysis of a Single Clone Exposed to I-SceI and 5-AzadC

A single clone DR-GFP, isolated from the HeLa cell pool, was transfected with I-SceI expression vector and treated with 5-AzadC as described in Figure 3. PCR of genomic DNA or cDNA was performed at different cycles (25 and 30) with a reference marker included (β-actin). The primers used were those described in Figure 1.

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Figure S3. DR-GFP Copy Number in Clones 2 and 3

(A) qPCR analysis of DNA extracted from clones 2 and 3 (see Figures 3 and 4) is shown. Reference curves were generated with 1, 3, and 10 pg of DR-GFP plasmid (2 pg represents approximately 1 copy/haploid genome in 300 ng of genomic DNA for a unique 15-kb DNA sequence). Shown here are curves generated with 1 pg of DR-GFP or with 300 ng of genomic DNA isolated from clones 2 and 3 or control transfected HeLa cells (right panels). PCR of genomic DNA mixed with 1 pg of DR-GFP indicates the sensitivity of our assay (left panels).

(B) qPCR carried out on 100 ng of total genomic DNA of clone 2 or 3 is presented. RT-PCR was performed on a 7500 RT-PCR System (Applied Biosystems) using the SYBR Green-detection system. Reference curves were generated for 1, 3, 5, 10, and 1,000 ng of DR-GFP. The mean value and standard deviation of the Rn of six replicates of 1 pg, 3 pg, and 1 ng of DR-GFP and 100 ng of genomic DNA are plotted (Rn: normalized reporter = emission intensity of SYBR Green/emission intensity of passive reference). The estimated copy number was 1–3 for clone 2 and 3–4 for clone 3.

(C) Southern blot analysis of NotI-cleaved DNA derived from clones 2 and 3 or HeLa cells is presented. The probe was a 1-kb fragment just upstream to the NotI site. The 15 kb corresponds to a tandem insertion of DR-GFP, whereas the 7.5- and 5.3-kb fragments correspond to two unique insertions at the upstream to the DR-GFP.

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Figure S4. Actual Frequency of Single Methylated CpG in Each Class of Molecules

Shown is actual frequency of single methylated CpG in each class of molecules in ES wild type (A), ES Dnmt1−/− (B), and HeLa (C) cells.

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Figure S5. Phosphorylated -γ-H2AX Foci at Individual DSBs Following I-SceI Exposure

DR-GFP HeLa cells were transfected with the I-SceI expression vector and stained with specific antibodies to phosphorylated (P)-γ-H2AX. Confocal micrographs depict P-γ-H2AX foci at 4 d after transfection with control plasmid (left) or I-SceI (right). Samples at 1 and 2 d did not show P-γ-H2AX staining.

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Figure S6. De Novo Methylation and LOH in Thyroid Tumors

DNA from three normal thyroid and five independent samples of anaplastic thyroid carcinomas was extracted and subjected to PCR analysis for four polymorphic microsatellite markers localized on human Chromosome 11 (centromere to telomere D11; S4117, S1784, S4183, S1350). LOH to the left or right of the CpG island between markers S4117 and S1784 was determined by PCR and is indicated as the ratio of normal versus tumor allele. "Not informative" indicates that the marker analyzed was identical in both alleles. In these cases LOH cannot be assessed. The CpG island is located upstream (−250 bp from the transcription start site) to the *PTPRJ* gene. DNA was subjected to bisulfite analysis and amplified with specific primers for the *PTPDJ* gene. Black and white circles represent methylated and unmethylated CpGs, respectively, in individual molecules, respectively. For each sample at least 10 DNA molecules were analyzed. WT indicates DNA derived from one normal sample, while M18, M29, M39, M43, and M107 DNA was derived from five anaplastic thyroid

carcinomas. M18 and M29 showed no LOH for the markers analyzed. Specifically, M18 showed no LOH (<5%) of the markers S4117, S1784, and S4183; S1350 was not informative. M29 showed no LOH (<5%) of the markers S1784 and S4183; S4117 and S1350 were not informative. In normal samples no significant CpG methylation was found 200 bp upstream or downstream to the segment shown in the figure.

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